In re Application of: Menachem RUBINSTEIN et al.
Serial No.: 10/560,232
Filed: March 24, 2006
Office Action Mailing Date: September 17, 2008

Examiner: Anna PAGONAKIS
Group Art Unit: 1614
Attorney Docket: 31129

REMARKS

Reconsideration of the above-identified application in view of the amendments above and the remarks following is respectfully requested.

Claims 1-46 are in this Application. Claims 12-46 have been withdrawn from consideration as being drawn to non-elected invention. Claims 1-11 have been examined on the merits, with ammonium trichloro(dioxoethylene-O,O')tellurate (AS101) as the elected species. Claim 1 has been amended herewith. Claims 2-5 have been canceled herewith.

The Application now comprises, after amendments, claims 1 and 6-46, of which claims are 1, 12, 24 and 35 in independent form.

35 U.S.C. §112, First Paragraph, Rejection

The Examiner has stated that claims 1-11 are rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the enablement requirement. The Examiner's rejection is respectfully traversed. Claim 1 has been amended. Claims 2-5 have been canceled.

Specifically, the Examiner has referred to the *Wands* factors and stated that the nature of the art is unpredictable, as Lillioja et al. teaches that *in vitro* studies cannot be relied upon for an accurate prediction for *in vivo* environments, and U.S. Patent No. 7,045,150 teaches the opposite of what is claimed by the Applicant, specifically that AS101 induces weight gain.

The Examiner has further stated that the specification provides no direction for determining the particular administration regimens (e.g., dosages, timing, administration routes, etc.) necessary to treat obesity, particularly in humans, that direction concerning treatment in humans is not found in the instant specification, that Applicant merely states that ob/ob mice are a predictive candidate for human obesity, and that no formulations or dosages or modes of administration are discussed.

The Examiner has therefore concluded that because of the unpredictability of the art and absence of experimental evidence commensurate in scope with the claims, the skilled artisan would not accept the assertion that the instantly claimed genus of

compounds could be predictably used as a treatment for obesity as inferred in the claims, that determining if any particular claimed compound would treat any particular cancerous disease state [sic] would require synthesis of the compound, formulation into a suitable dosage form, and subjecting it to clinical trials or to testing, and that this is undue experimentation given the limited guidance and direction provided by Applicant.

Applicants wish to note that the Examiner's statements are unclear. Specifically, the Examiner initially refers specifically to treatment of obesity with AS101 (the elected species) as the scope of the subject matter under examination (see, for example, page 3, final sentence, of the present Office Action, as well as page 5 therein:

> *"The breadth of the claims*
>
> *The claims encompass the treatment of obesity comprising AS101".*

However, the Examiner's concluding remarks refer to a plurality of compounds. See page 5, penultimate paragraph, of the Office Action, "the instantly claimed genus of compounds"; and page 6, first full paragraph, therein, "Determining if any particular claimed compound would treat any particular cancerous disease state [sic]".

Applicants' following remarks are provided under the assumption that the Examiner's rejection relates to a method of treating obesity comprising administering the elected species (i.e., AS101). Applicants believe that it would be improper to issue a Final Office Action if this assumption is incorrect, due to the lack of clarity of the outstanding Office Action.

Applicants traverse the Examiner's rejection for the following reasons, which are discussed in detail hereinbelow:

a) The Examiner has overstated the unpredictability of the art, particularly in view of the efficacy data presented in the instant Application; and

b) The specification provides direction and guidance regarding administration regimens, contrary to the Examiner's assertions.

Re: unpredictability of the art:

The Examiner has referred to Lillioja et al., who teach that *in vitro* measurement of fat cell lipolysis cannot be used to directly predict *in vivo* free fatty acid metabolism.

However, the instant Application provides experimental evidence of effectiveness of the claimed invention from two *in vivo* experiments (see, for example, page 21-22, Examples 1-3, therein). Hence, the relevance of the problems with *in vitro* measurements is unclear to Applicants.

Furthermore, the *in vitro* results presented in the instant application describe reduced adipocyte generation (see page 22, Example 4, therein). In sharp contrast, Lillioja et al. refers to *in vitro* lipolysis measurements, which do not relate to the *in vitro* model presented in the instant Application.

Moreover, the teachings of Lillioja et al. do not suggest anything regarding the predictability of treatment of obesity in particular, as it is well known in the pharmaceutical arts that *in vivo* experimental results are generally more predictive than *in vitro* experimental results.

In this regard, Applicants further note the following: The effectiveness of the claimed invention has been demonstrated in two *in vivo* animal models, specifically, an *ob/ob* mouse model and a diet-induced obese C57BL/6 mouse model. Both of these animal models are well known art-recognized models of obesity. See, for example, Shore [*J. Appl. Physiol.* 2007, 102:516-528], particularly page 517, first paragraph, and page 518, first paragraph, therein; Buettner et al. [*Obesity (Silver Spring)* 2007, 15:798-808], particularly the Abstract thereof; and Collins et al. [*Physiol. Behav.* 2004, 81:243-248], particularly the Abstract thereof. Copies of the aforementioned references are submitted herewith.

MPEP §2164.02 states that a model (including an *in vitro* model) constitutes a "working example" if the model is recognized as correlating to a specific condition, unless the Examiner has evidence that the model does not correlate. Even with such

evidence, the Examiner must weigh the evidence for and against correlation. *In re Brana*, 51 F.3d 1560, 1566, 34 USPQ2d 1436, 1441 (Fed. Cir. 1995).

MPEP §2164.02 further states that the initial burden is on the Examiner to give reasons for a conclusion of lack of correlation for an *in vitro* or *in vivo* model. A rigorous or an invariable exact correlation is not required. *Cross v. Iizuka*, 753 F.2d 1040, 1050, 224 USPQ 739, 747 (Fed. Cir. 1985).

See also *Ex parte PAUL P. LATTA* (Appeal 2007-1152; Application No. 10/660,924), which states:

> *"The Examiner's position appears to be that because mouse models don't always work, they cannot be relied upon to enable a specification – there being always a degree of uncertainty about whether the treatment will prove to be effective in humans. In our opinion, this is not a reasonable standard by which to measure enablement....*
>
> ***We see no reason why enablement of a method claim whose scope includes humans should likewise require human testing.***"

Applicants therefore submit that since the problems with *in vitro* lipolysis measurements, as taught by Lillioja et al., are not relevant to the predictability of the art, considering the teachings of the instant Application, the Examiner has not shown lack of correlation between the enabling models presented in the instant Application and the claimed invention.

U.S. Patent No. 7,045,150 teaches that tellurium-containing compounds enhance growth of immature poultry (see, for example, column 1, lines 18-21, and column 3, lines 27-35, therein).

In sharp contrast, the claimed invention is of a method of treating obesity.

Although U.S. Patent No. 7,045,150 makes reference to "cumulative weight gain" as an indication of growth enhancement, it would be apparent to one of ordinary

skill in the art that weight gain in a growing immature individual is unrelated to weight gain which results in obesity. Thus, for example, weight gain is routinely measured in children as an indicator of growth, and is not in any way construed as being an indicator of obesity. Rather, obesity is typically defined according to weight adjusted according to size (see, for example, page 1, lines 15-18, of the instant application).

Moreover, U.S. Patent No. 7,045,150 neither teaches nor suggests that any of the poultry described therein are obese.

As the weight gain described in U.S. Patent No. 7,045,150 is completely unrelated to obesity, it is submitted that U.S. Patent No. 7,045,150 does not contradict in any way, let alone teach the "opposite" of, the claimed invention.

It is therefore submitted that none of the cited references has any relevance in showing the unpredictable nature of the art, particularly in view of the in vitro and in vivo studies presented in the instant Application.

The Examiner has therefore not provided any evidence suggesting that the *in vivo* and *in vitro* models presented in the instant application do not correlate to obesity. Thus, as discussed hereinabove, Lillioja et al. merely refers to an *in vitro* lipolysis model which is not mentioned in the instant application, and U.S. Patent No. 7,045,150 relates to growth of immature poultry, which is unrelated to the abovementioned obesity models. The Examiner has not made reference to any other sources of evidence.

It is therefore concluded that the instant Application provides multiple working examples demonstrating the effectiveness of the claimed invention, in accordance with the above-indicated MPEP standards.

Re: amount of direction or guidance provided in the specification:

Applicants respectfully note that dosage, timing and routes of administration are discussed in detail in the instant application (see, for example, the description spanning from page 19, line 6, to page 20, line 15, therein) and exemplified in two art-

recognized *in vivo* animal models for which dosage, timing and route of administration are specified (see, for example, page 21-22, Examples 1-3, therein).

Applicants further note that the Examiner has not provided any explanation why the abovementioned direction and guidance may be inadequate for enabling the claimed invention.

Hence, the Examiner's statements that there is no direction or guidance for determining the particular administration regimens (e.g., dosages, timing, administration routes, etc.), that direction concerning treatment in humans is not found in the instant specification, and that no formulations or dosages or modes of administration are discussed, are unclear to Applicants.

As dosage, timing and routes of administration are discussed in detail in the instant application, and further since multiple working examples are provided therein, Applicants believe that a person skilled in the art should not be engaged in undue experimentation, with no assurance of success, when practicing the claimed invention.

Applicants therefore submit that the claimed invention complies with the enablement requirements of 35 U.S.C. §112, first paragraph.

Notwithstanding the above, Applicants have chosen, in order to expedite prosecution, to amend claim 1 so as to read on ammonium trichloro(dioxoethylene-O,O')tellurate (AS101).

Consequently, claims 2-5, which recited limitations now included in amended claim 1, have been canceled.

As argued hereinabove, Applicants strongly believe that the instant specification is enabling with regard to treating obesity with AS101.

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In view of the above amendments and remarks it is respectfully submitted that claims 1 and 6-11 are now in condition for allowance. A prompt notice of allowance is respectfully and earnestly solicited.

Respectfully submitted,

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Date: March 16, 2009

Enclosures:

- Petition for Extension of Time (Three Months); and
- References:
  a) Shore, S.A., *J. Appl. Physiol.* 2007, 102:516-528;
  b) Buettner, R. et al., *Obesity (Silver Spring)* 2007, 15:798-808;
  c) Collins, S. et al., *Physiol. Behav.* 2004, 81:243-248; and
  d) *Ex parte PAUL P. LATTA* (Appeal 2007-1152; Application No. 10/660,924)

Invited Review

J Appl Physiol 102: 516–528, 2007;
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Obesity and asthma: lessons from animal models

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Shore SA. Obesity and asthma: lessons from animal models. *J Appl Physiol* 102: 516–528, 2007; doi:10.1152/japplphysiol.00847.2006.— Epidemiological data indicate that obesity is a risk factor for asthma. These data are supported by observations in several murine models of obesity. *Ob/ob*, *db/db*, and *Cpe^{fat}* mice each exhibit innate airway hyperresponsiveness, a characteristic feature of asthma. These mice also respond more vigorously to common asthma triggers, including ozone. Here we discuss the implications of these data with respect to several mechanisms proposed to explain the relationship between obesity and asthma: *1*) common etiologies; *2*) comorbidities; *3*) mechanical factors; and *4*) adipokines. We focus on the role of adipokines, especially TNF-α, IL-6, leptin, and adiponectin. Understanding the mechanistic basis for the relationship between obesity and asthma may lead to novel therapeutic strategies for treatment of the obese asthmatic subject.

mice; leptin; adiponectin; tumor necrosis factor-α; interleukin-6; adipokines

ACCORDING TO THE US CENTERS for Disease Control, ~65% of the US population is either overweight or obese. The prevalence has more than doubled during the last 20 years (www.cdc.gov/nccdphp/dnpa/obesity/trend/index.htm) and continues to rise. Obesity is a known risk factor for atherosclerosis, hypertension, Type 2 diabetes, and some forms of cancer (33). Obesity is also a risk factor for asthma. Data supporting a relationship between obesity and asthma include over 30 cross-sectional studies performed in adults and children of multiple ethnicities throughout the world [for a complete list, see recent reviews (34, 115)]. With relatively few exceptions, each of these studies indicates a greater prevalence of asthma in the obese. While such cross-sectional studies cannot sort out the direction of causality, data from 13 prospective studies in adults, adolescents, and children indicate that obesity antedates asthma [see Shore and Johnston (115)]. In these studies, several hundred thousand individuals initially free of asthma were followed for periods varying from 2 to 21 yr. In aggregate, the results indicate that the relative risk of incident asthma increases with body mass index (BMI) and that even overweight conveys some increased risk. Obesity may also increase disease severity in subjects who already have asthma (1). In addition, obesity appears to alter the efficacy of standard asthma medications (98).

The observations that both weight loss and weight gain impact asthma provide additional evidence of a relationship between obesity and asthma. The impact of weight gain can be marked. For example, Camargo et al. (14) reported that women who gained >25 kg since age 18 yr had a relative risk for incident asthma almost five times that of women whose weight remained stable. In contrast, surgically induced weight loss results in significant improvements in all asthma outcomes, including prevalence, severity, use of asthma medications, and hospitalizations for asthma (27, 121, 124). Studies of diet-induced weight loss in obese asthmatic subjects also report improvements in flow rates (45, 125).

The reason for the relationship between obesity and asthma has not been established, although several possibilities have been suggested (112, 115, 142, 143) (Fig. 1). It has been hypothesized that obesity and asthma share a common etiology, such as a common genetic predisposition, or common effects of in utero conditions. It may also be that obesity per se is not the culprit. Instead, comorbidities of obesity, such as gastroesophageal reflux or sleep-disordered breathing, may provoke or worsen asthma. The obese breathe with small tidal volumes at low absolute lung volume, and both of these factors could be expected to increase airway obstruction. Finally, it is now increasingly appreciated that there are alterations in the endocrine function of adipose tissue in obesity. Obesity-related changes in adipose-derived hormones, cytokines, and other factors may also play a role in the relationship between obesity and asthma.

To examine the mechanistic basis for the relationship between obesity and asthma, we set out to determine whether we could model the relationship between obesity and asthma in mice. Here we review the various murine models of obesity that have been studied and the pulmonary phenotype of these mice, a phenotype that includes both innate airway hyperresponsiveness (AHR), a defining characteristic of asthma, as well as increased responses to ozone (O_3) and allergen, two common triggers for asthma. We also review what we have learned from these mice about the likely mechanistic basis for the relationship between obesity and asthma.

ANIMAL MODELS OF OBESITY

The pulmonary phenotype of several types of obese mice has been characterized (60, 81, 92, 103, 116, 129, 130). A brief description of the cause and nature of the obesity in each of these models is presented below, and their phenotypes are summarized in Table 1. We also describe several other murine models that may prove useful in unraveling the relationship between obesity and asthma.

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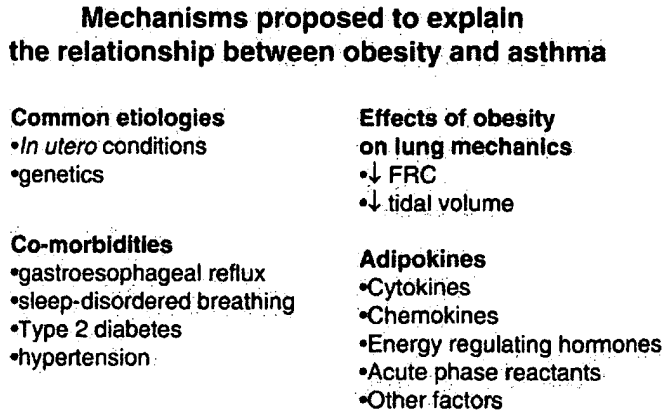

Fig. 1. Potential mechanisms whereby obesity may be associated with asthma. FRC, functional residual capacity.

ob/ob Mice

Leptin is a satiety hormone that is synthesized by adipocytes, and serum leptin increases with the mass of adipose tissue (23, 82, 157). The *ob/ob* mouse is the result of a spontaneous mutation that arose in the leptin gene in a colony of mice at Jackson Laboratories. A single base pair substitution in *codon 105* of the leptin gene results in a premature stop codon. In the absence of leptin, *ob/ob* mice eat excessively and are already obese at 4 wk of age. By 8 wk, they weigh at least twice as much as wild-type controls. The increased body weight is entirely the result of an increase in fat mass (11). The *ob/ob* mice also have a low resting metabolic rate, hypothermia, hypoactivity, hyperinsulinemia, hyperglycemia, increased serum corticosterone, increased fasting triglycerides and cholesterol, and are infertile (70, 88). Repletion of *ob/ob* mice with exogenous leptin reverses this phenotype (46, 97).

db/db Mice

Leptin suppresses appetite and increases metabolism by binding to leptin receptors in the hypothalamus. Several leptin receptor isoforms are generated by alternative splicing of the gene (79). In the *db/db* mouse, a mutation in the cytoplasmic domain of the long form of the leptin receptor, Ob-R_b, results in loss of expression of this isoform (18). Ob-R_b is the only leptin receptor isoform that contains the signal transducer and activator of transcription (STAT)-3 binding site, and leptin-induced STAT-3 activation, an event required for leptin's effect on satiety and metabolism, is absent in *db/db* mice (40, 131). Thus *db/db* mice are similar to *ob/ob* mice in many respects: *db/db* mice overeat, gain weight, are hypothermic, and are inactive. Like *ob/ob* mice, they weigh more than twice as much as wild-type controls at 8 wk of age. Like *ob/ob* mice, *db/db* mice are also hyperinsulinemic, hyperglycemic, hyperlipidemic, and they have increased plasma cholesterol (88, 110). However, while the *db/db* mouse lacks Ob-R_b, other isoforms are expressed (40, 48). These short forms of the leptin receptor have truncated (Ob-R_a, Ob-R_c, Ob-R_d) or absent (Ob-R_e) cytoplasmic domains; are expressed in peripheral tissues, especially in lung tissue (79); and are capable of some types of signaling, even though they lack the ability to activate STAT-3. Hence there can be subtle differences between *ob/ob* and *db/db* mice, including differences in their pulmonary phenotype (81).

Cpe^{fat} Mice

Carboxypeptidase E (Cpe) is required for processing of insulin, enkephalins, neurotensin, and other neuropeptides (36, 67). A missense mutation of the enzyme in *Cpe^{fat}* mice results in the production of a protein that is inactive because of replacement of Ser^{202} by Pro^{202} (71). Cpe cleaves and processes neuropeptides, such as corticotropin-releasing factor and neuropeptide Y, that mediate eating behaviors and energy expenditure, and the absence of these actions of the enzyme promotes obesity in *Cpe^{fat}* mice (70). Obesity develops more slowly than in *ob/ob* or *db/db* mice, but it can be quite marked (21). On the C57BL/6 background, *Cpe^{fat}* mice weigh ~20, 50, and 75% more than wild-type controls at 7, 10, and 15 wk of age, respectively (60, 62). As in the *ob/ob* and *db/db* mice, the excess weight is accounted for by increases in body fat, and the mice also have high fasting cholesterol (88). Some reports indicate increased fasting triglyceride levels in these mice as in the *ob/ob* and *db/db* mice (89), while others report no increase (88). As in *ob/ob* and *db/db* mice, hyperinsulinemia is observed (21, 87), but the great majority of the insulin measured in the serum of these mice is not insulin but proinsulin, likely because Cpe is required for processing of insulin. Proinsulin has low biological potency (71). Hyperglycemia, if present, is milder than in *ob/ob* and *db/db* mice (21).

Table 1. *Phenotypes of obese mouse*

	Ob/ob	*Db/db*	*Cpe^{fat}*	DIO	Reference No.
Obesity	Massive	Massive	Moderate	Milder	11, 21, 60, 70, 92, 97
Hyperinsulinemia	Yes	Yes	Yes*†	Yes	21, 52, 70, 87, 88, 93, 110
Hyperglycemia	Yes	Yes	Yes* (mild)	Yes	21, 52, 70, 88, 93, 110
Increased plasma cholesterol	Yes	Yes	Yes*	Yes	75, 88
Innate AHR	Yes	Yes	Yes	Yes	60, 81, 103, 116 and unpublished observations
Small lungs	Yes	Yes	No	No	60, 81, 116, 130
O_3-induced AHR	Greater than wild type	Greater than wild type	Greater than wild type	N/A	60, 81, 103, 116
O_3-induced inflammation	Greater than wild type	Greater than wild type	Greater than wild type	Greater than wild type	60, 81, 116

AHR, airway hyperresponsiveness; O_3, ozone; DIO, diet-induced obesity; N/A, not assessed. *Available data are from *Cpe^{fat}* mice on a C57BL/Ks background. Otherwise, all data are from mice on a C57BL/6 background. †Most of the insulin is proinsulin.

Diet-Induced Obesity

Weanling C57BL/6 mice fed a diet in which 45 or 60% of calories are derived from fat (predominantly in the form of lard) develop obesity (11, 92, 137). After 20 wk on a 60% fat diet, body weight is ~25% greater than for age-matched mice fed a diet in which only 10% of calories derive from fat. After 32 wk on the diet, weight is increased by ~45% (unpublished observations). Most of the increase in body weight is the result of an increase in fat mass (11), similar to *ob/ob* mice. Mice rendered obese by high-fat diet, like *ob/ob* and *db/db* mice, are also hyperglycemic and insulin resistant (52, 93, 110, 126). Plasma cholesterol is also elevated in this model (75).

Other Murine Models of Obesity

Mice with other forms of genetic obesity exist (15, 21, 53), but their pulmonary phenotype has not been characterized. Tubby mice (21) and G protein-coupled receptor-7-deficient mice (53) display mature-onset obesity that may be useful in separating effects of obesity on lung development from other effects that lead to altered airway function (see below). G protein-coupled receptor-7 is the receptor for neuropeptides B and W that are involved in eating behavior (128), while *tub* is a gene expressed at high levels in regions of the hypothalamus involved in eating behavior (107), whose precise function is still not defined. Since hyperinsulinemia is milder in tubby mice than in *ob/ob* and *db/db* mice, and hyperglycemia is not observed (21), tubby mice may also prove useful in determining whether insulin resistance or hyperglycemia contribute to the lung phenotype observed in obese mice. Jackson Laboratories has also developed some congenic strains of mice that differ in their susceptibility to Type 2 diabetes and obesity that may be helpful in understanding the role of hyperglycemia for asthma (72, 73, 101). In addition to genetically obese mice, methods also exist for inducing obesity in otherwise normal mice. For example, injecting mice with gold-thioglucose selectively ablates glucose-responsive neurons in the ventromedial hypothalamic nucleus, causing hyperphagia and obesity (53). Such methods may prove useful for inducing obesity in genetically altered mice.

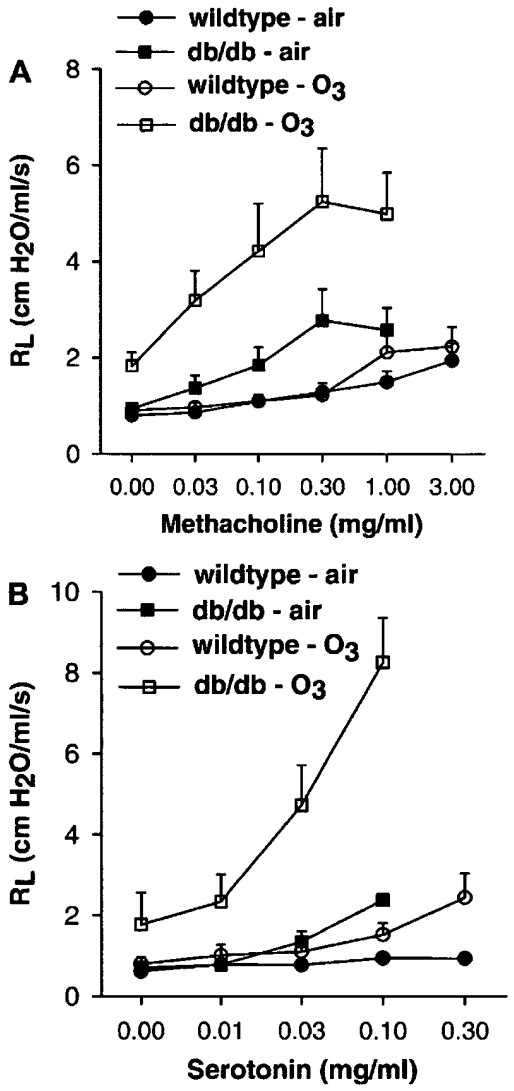

Fig. 2. Changes in lung resistance (RL) induced by intravenous methacholine (*A*) or serotonin (*B*) in wild-type (C57BL/6) and *db/db* mice exposed to air or ozone (O_3) [2 parts/million (ppm) for 3 h]. Measurements were made 24 h after exposure. Results are means ± SE of data from 6–11 mice in each group. [Adapted from Lu et al. (81) and reproduced with the permission of the American Physiological Society.]

OBESE MICE EXHIBIT INNATE AHR

Airway responsiveness to intravenous methacholine is increased in *ob/ob*, *db/db*, and *Cpe^{fat}* mice (60, 81, 103, 116), indicating that it is a common feature of murine obesity. In these obese mice, AHR is nonspecific, since increased responses to serotonin are also observed (81) (Fig. 2). Nonspecific AHR to multiple bronchoconstricting agonists is also a feature of human asthma. We have also examined airway responsiveness in mice with diet-induced obesity (unpublished observations). By 23 wk of age, when weight gain is still moderate (25% increase), no AHR is observed in mice on diets in which 60% of calories derive from fat, but by 35 wk, when more substantial weight gain has occurred (45% increase), airway responsiveness is increased, suggesting that weight gain must be fairly substantial before AHR is manifest. By comparison, *ob/ob*, *db/db*, and *Cpe^{fat}* mice weighed 175, 150, and 85% more than wild-type controls, respectively, when AHR was observed (60, 81, 116). AHR has also been associated with obesity in three large epidemiological studies (16, 19, 77), although this association is not consistently observed (34, 115). Most of the studies in which AHR was not observed to correlate with BMI involved smaller numbers of subjects and may have been less powered to detect differences, especially if substantial increases in body weight are required for the development of AHR.

We used changes in pulmonary resistance (RL) to assess responses to methacholine in obese mice. While RL includes contributions from both the airways and the lung tissue, measurements made in both *ob/ob* (103) and *Cpe^{fat}* mice (60) indicate that the increased responses to methacholine observed in obese mice are the result of differences in the airways. Changes in lung tissue resistance contribute very little to changes in RL induced by intravenous methacholine in either obese or lean mice (60, 103).

While we do not currently know the mechanistic basis for the AHR observed in obese mice, we have ruled out several possible explanations. We have not observed any overt inflammation in the lungs of unchallenged obese mice, nor are there differences in the number of macrophages harvested from obese vs. lean mice by bronchoalveolar lavage (BAL) (60, 81). As discussed in more detail in MECHANISTIC BASIS FOR THE RELATIONSHIP BETWEEN OBESITY AND ASTHMA below, obesity-related alterations in functional residual capacity (FRC) and tidal volume may play a role in the relationship between obesity and asthma in spontaneously breathing humans, but additional factors must be important for the innate AHR observed in mice, since all measurements of pulmonary mechanics were made in open-chested mice, mechanically ventilation at a fixed positive end-expiratory pressure (PEEP) and a fixed tidal volume. Since AHR was observed both in *ob/ob* and *db/db* mice with leptin or leptin receptor deficiency, and in Cpe^{fat} mice and mice with diet-induced obesity that have marked increases in serum leptin (60, 92, 137), it is unlikely that leptin is involved, even though leptin has the potential to augment airway responsiveness (118). As discussed in more detail in MECHANISTIC BASIS FOR THE RELATIONSHIP BETWEEN OBESITY AND ASTHMA below, other adipokines may be involved in the innate AHR observed in obese mice. It is conceivable that hyperinsulinemia, which is marked in *ob/ob* and *db/db* mice, may contribute to the innate AHR observed in these strains. However, Cpe^{fat} mice also exhibit innate AHR. In these mice, hyperinsulinemia, although present, is the result of increased proinsulin, which is largely inactive, as described above. We also cannot rule out the possibility that hyperglycemia contributes to AHR, since all strains studied to date exhibit this phenotype to some extent (see above). There is currently increased interest in the hypothesis that increased oxidative stress, which can be a consequence of hyperglycemia, contributes to many aspects of the obese phenotype (50, 109), and oxidative stress has also been linked to asthma (64).

OBESE MICE HAVE INCREASED RESPONSES TO ACUTE O_3 EXPOSURE

Exposure to O_3, a common air pollutant, is a trigger for asthma. Hospital admissions for asthma are higher on days of high ambient O_3 concentrations (32, 134), and, in children, O_3 increases asthmatic symptoms even at concentrations below the US Environmental Protection Agency standard (39). O_3 causes lung injury and an inflammatory response that includes the generation of prostanoids, cytokines, and chemokines, as well as an influx of neutrophils into the lungs (25, 56–60, 65, 81, 113). O_3 also causes AHR (60, 81, 90, 156). Both O_3-induced AHR and O_3-induced inflammation are likely to contribute to the ability of O_3 to exacerbate asthma.

Obesity impacts the effects of O_3 in the lung. Pulmonary mechanics and airway responsiveness were measured 24 h after the cessation of acute O_3 exposure (2 parts/million for 3 h) in *ob/ob* mice (103, 116), *db/db* mice (81), Cpe^{fat} mice (60), and their lean age- and sex-matched C57BL/6J wild-type controls. O_3 exposure increased R_L in obese mice, regardless of the modality of obesity, whereas O_3 had no effect on R_L in lean mice. In addition, O_3 exposure caused much more robust changes in airway responsiveness in obese than in lean mice. In *ob/ob* and Cpe^{fat} mice, we only measured responses to methacholine, but in *db/db* mice, greater O_3-induced AHR was observed, regardless of whether methacholine and serotonin was used as the bronchoconstricting agonist (Fig. 2), confirming the nonspecific nature of the AHR. A recent preliminary report also indicates greater effects of acute O_3 exposure on pulmonary mechanics with increasing BMI in human subjects, especially women (6).

O_3-induced injury and inflammation were also greater in *ob/ob* and *db/db* mice than in lean controls (60, 81, 116). Some investigators have reported that O_3-induced AHR is mechanistically linked to certain aspects of O_3-induced inflammation (20, 58, 117), although this is not always the case (74, 156). Hence it is possible that the augmented O_3-induced AHR observed in obese mice is the result of their greater inflammatory response. To examine changes in the pulmonary inflammatory response to O_3 over time with the development of obesity, we also exposed 7-, 10-, and 14-wk-old Cpe^{fat} mice and their age-matched wild-type controls to O_3 (60, 62). Body weight averaged 20, 50, and 75% more, respectively, in these Cpe^{fat} mice than in age-matched wild-type mice. O_3-induced airway inflammation was greater in Cpe^{fat} than wild-type mice, regardless of age (see Fig. 3 for data from 14-wk-old mice). Thus even a relatively moderate 20% increase in body weight is sufficient to increase the effects of O_3 in mice, whereas more substantive changes appear to be required for effects on AHR (see above). Notably, serum leptin was substantially elevated at as early as 7 wk of age in Cpe^{fat} mice compared with age-matched controls, whereas changes in other measured indexes of systemic inflammation were not changed until 10 wk of age. Leptin can augment some aspects of the pulmonary response to O_3 (116), but there must be important effects of obesity in addition to changes in serum leptin, since most of the inflammatory moieties induced by O_3 are increased in *ob/ob* and *db/db* mice (81, 116), just as they are in Cpe^{fat} mice (60).

We initially considered the possibility that an increased inhaled dose of O_3 was responsible for the increased response to O_3 observed in obese mice. The inhaled dose of O_3 is the product of O_3 concentration, exposure time, and minute ventilation (144). O_3 concentration and exposure time were identical in obese and lean mice. While there was greater minute ventilation in *db/db* mice than wild-type mice during O_3 exposure (81), this was not true in *ob/ob* mice or in Cpe^{fat} mice (60, 81, 116). An additional issue related to dose is the small lung size of the *ob/ob* and *db/db* mice (see MECHANISTIC BASIS FOR THE RELATIONSHIP BETWEEN OBESITY AND ASTHMA below). Even if the inhaled dose of O_3 was the same in obese and lean mice, the dose per gram of lung tissue was higher in the *ob/ob* and *db/db* mice than in wild-type controls, because their lung mass was smaller (116). We do not think this accounts for the greater inflammatory responses to O_3 observed in obese mice, because similar increased responses were observed in Cpe^{fat} mice (60) and in mice with diet-induced obesity (unpublished observations). Both of these types of obese mice have lungs of normal mass.

OBESITY AND ALLERGIC RESPONSES

Atopy is an important risk factor for asthma, especially in children. What have animal models taught us about the effects of obesity on pulmonary responses to allergen challenge? Mito et al. (85) sensitized and challenged mice with diet-induced

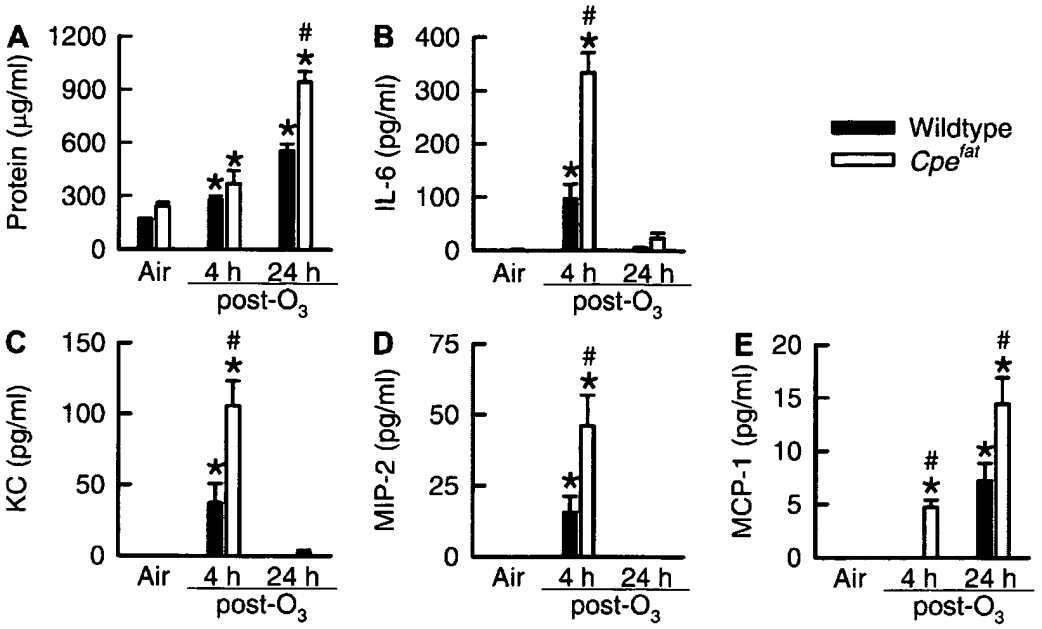

Fig. 3. The concentration of total protein (*A*), IL-6 (*B*), KC (*C*), macrophage inflammatory protein (MIP)-2 (*D*), and monocyte chemoattractant protein (MCP)-1 (*E*) in the bronchoalveolar lavage fluid (BALF) of wild-type (C57BL/6) and *Cpe*^{fat} mice either 4 or 24 h after exposure to either room air or O_3 (2 ppm for 3 h). n = 6–12 Mice for each group. *$P < 0.05$ compared with genotype-matched air-exposed controls. #$P < 0.05$ compared with O_3-exposed, wild-type (C57BL/6) mice with an identical exposure. [Adapted from Johnston et al. (60) and presented with the permission of the American Physiological Society.]

obesity using ovalbumin (OVA). In the absence of sensitization, splenocytes from the obese mice had impaired proliferative responses compared with that from lean controls. However, following OVA sensitization and challenge, splenocyte proliferation, IL-2 production, and mast cell numbers were increased, while OVA-specific IgG_1 and IgE were decreased in obese vs. lean mice. These authors did not measure airway responsiveness, but preliminary data from our laboratory indicate increased OVA-induced AHR in *ob/ob* mice vs. wild-type controls, even though there are no differences in T helper type 2 (Th2) airway inflammation (104). In contrast to the data from Mito et al. (85), we also observed increased effects of OVA challenge on IgE in *ob/ob* mice (104). The reason for this difference between our results and those of Mito et al. is not apparent, but it may be related to the modality of obesity. Although they did not examine obesity per se, Yeh and Huang (151) examined the impact of cholesterol on airway responses to OVA sensitization and challenge. They observed increased numbers of BAL fluid eosinophils and lymphocytes and increased levels of IL-5 in mice fed diets supplemented with cholesterol vs. chow-fed controls. Together, these results indicate that obesity and/or dietary constituents elevated in obesity can augment some but not all responses to allergen challenge.

Interestingly, results of studies of the effect of obesity on atopy have also been inconsistent [see Shore and Johnston for review (115)]. For example, some investigators have reported important effects of obesity on asthma in nonatopic, but not in atopic, subjects, and others have reported significant effects of BMI on asthma but not on other atopic diseases. In contrast, there have been some reports of increases in atopy with obesity, mostly in children. Since the onset of atopy usually occurs early in life, weight gain in early life may be more important for the development of atopy than weight gain as an adult. In this respect, mice in which obesity develops very early in life, such as *ob/ob* and *db/db* mice, may turn out to be more suitable as models for the interaction of obesity with atopy than other types of obese mice that become obese much later in life.

MECHANISTIC BASIS FOR THE RELATIONSHIP BETWEEN OBESITY AND ASTHMA

As described above, several mechanisms have been proposed to account for the relationship between obesity and asthma (Fig. 1). Below we summarize these mechanisms and describe, where available, evidence from animal models that addresses each one.

Epiphenomenon

When the epidemiological data demonstrating a relationship between obesity and asthma were first reported, one consideration was that this relationship was an epiphenomenon (14). It was suggested that the true relationship was between exercise and asthma or between diet and asthma. Indeed, increases in certain dietary constituents and lack of exercise can both lead to obesity. Several prospective studies controlling for exercise have since discounted the likelihood that lack of exercise is responsible for the relationship between obesity and asthma (5, 14, 91). Data from mice indicate that a relationship between obesity and an asthma-like phenotype in the lung can also exist in the absence of differences in diet. In the studies described above, mice with genetic obesity (*ob/ob*, *db/db*, and *Cpe*^{fat} mice) ate exactly the same diet as their lean wild-type controls. They just ate more of it.

Common Etiologies

As described above, it has been proposed that obesity and asthma share a common etiology, for example, a common genetic predisposition, or common effects of in utero condi-

tions, and that the observed increases in the prevalence and incidence of asthma in the obese arise from this common predisposition (142). If so, there must be different factors that contribute to the relationship between obesity and an asthma-like pulmonary phenotype in mice, since the genetically obese mice in these studies differed from their wild-type controls at a single gene, a gene that was different in each model. In addition, *ob/ob*, *db/db*, and *Cpe^{fat}* mice are all infertile. Consequently, their mothers are all heterozygous and lean, and their in utero conditions are unlikely to differ from wild-type mice.

Comorbidities

It is also possible that comorbidities of obesity, such as gastroesophageal reflux or sleep-disordered breathing, may provoke or worsen asthma. Each of these conditions is known to affect asthma (44). To our knowledge, the presence of gastroesophageal reflux has not been assessed in any of the murine models of obesity described above. However, obese mice do have disordered sleep. *Ob/ob* mice have disrupted sleep architecture characterized by increased numbers of arousals and increased stage shifts compared with wild-type mice (69), and both *ob/ob* mice and mice with diet-induced obesity have increased amounts of 24-h non-rapid eye movement sleep (55, 69). Control of breathing during sleep is also altered in *ob/ob* mice, but is restored by exogenous leptin administration (92), indicating that it is the result of leptin deficiency, not some other aspect of obesity. In contrast, exogenous leptin does not reverse the increased O_3-induced inflammation observed in *ob/ob* mice (116), suggesting that the latter phenotype is not the result of effects of sleep-disordered breathing. Similarly, control of breathing during sleep is not affected in mice with diet-induced obesity (92), even though these mice do have innate AHR and increased responses to O_3 (unpublished observations). Hypercholesterolemia is a risk factor for asthma, but it appears to act independently of obesity (2). Hypertension and Type 2 diabetes are also important comorbid conditions with obesity, but little is known about the impact of these conditions on asthma.

Mechanical Factors

Both static and dynamic mechanical factors extant in the lungs of obese subjects have the capacity to aggravate airway obstruction (114, 115). The act of breathing stretches airway smooth muscle, causing actin-myosin cross bridges to detach, leading to bronchodilation (35, 43). Obese humans (108) and obese mice (115) breathe spontaneously with lower than normal tidal volumes, which would compromise this potent bronchodilating mechanism. Because of the load imposed by the increased abdominal and chest wall mass, FRC is also reduced in obesity (150). Absolute lung volume is a major determinant of airway diameter (26), and a lower FRC may unload the airway smooth muscle, allowing it to shorten excessively when activated. These mechanical factors may play a role in spontaneously breathing obese subjects. They may also be compounded by obesity-related changes in the compliance of the airways, which would further reduce the ability of tidal stretching to dilate the airways. Bergeron et al. (9) and Komakula et al. (66) each reported a decreased ability of a deep inspiration to dilate the airways of obese vs. lean asthmatic subjects. However, differences in tidal strain are unlikely to account for the AHR observed in obese mice, because measurements of pulmonary mechanics were made with the mice mechanically ventilated using a tidal volume that was the same for obese and lean mice. Nevertheless, we cannot rule out the possibility that breathing at low tidal volume for extended periods of time causes adaptations in the airway smooth muscle that are not easily reversed. We also removed the influence of the chest wall mass on absolute lung volume by measuring R_L with the mice open chested and at a fixed PEEP. However, it is important to note that breathing at low absolute lung volume for several hours increases airway resistance, even after lung volume has been restored (24). Another important caveat must be considered with respect to lung volume. In *ob/ob* and *db/db* mice, end-expiratory lung volume is reduced, even when measured in open-chested animals with a fixed PEEP (81, 116, 130). The reduction in lung volume appears to be the result of decreased lung growth, since lung mass is also reduced (116). The small lungs of *ob/ob* and *db/db* mice may result from lack of the effects of leptin, which can act as a growth factor in the lung (8, 135). Alternatively, since obesity develops very early in these mice, it may be that the increased fat mass restricts lung growth during development. Large amounts of intrathoracic fat are observed even in 8-wk-old *ob/ob* and *db/db* mice. Alterations in lung or airway anatomy resulting from effects of obesity/leptin deficiency on lung development may contribute to the AHR observed in *ob/ob* and *db/db* mice, but the observation that lung size is normal in *Cpe^{fat}* mice (60) and in mice with diet-induced obesity (unpublished observations), which also exhibit AHR, does not support this hypothesis.

Adipokines

In humans, even in the absence of any overt inflammatory stimulus, the obese state is characterized by increases in the serum levels of multiple cytokines, chemokines, and soluble cytokine receptors, all of which decline with weight loss (Fig. 4). These include, but are not limited to, TNF-α, IL-1, IL-6,

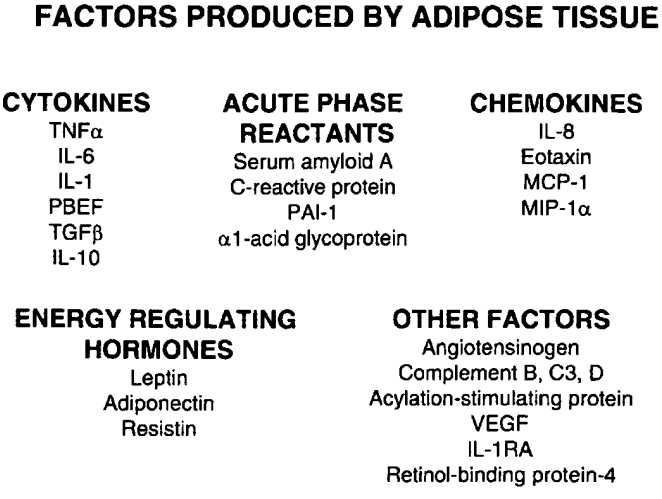

Fig. 4. Some of the proteins produced by adipose tissue (adipokines). IL, interleukin; TNF, tumor necrosis factor; PBEF, pre-B-cell colony-enhancing factor; TGF, transforming growth factor; PAI, plasminogen activator inhibitor; VEGF, vascular endothelial growth factor; IL-1RA, interleukin-1 receptor antagonist.

soluble TNF receptor 1 (sTNFR1), monocyte chemoattractant protein-1, eotaxin, IL-8, and macrophage inflammatory protein (MIP)-1α (3, 49, 54, 109, 127, 138, 139). Circulating leukocytes are also increased (155). Accordingly, the term "low-grade systemic inflammation" has sometimes been used to describe the obese state. Obese mice exhibit a similar low-grade systemic inflammation (29, 111, 149). The source of many of these inflammatory moieties appears to be the adipose tissue itself. For example, the mRNA expression of many inflammatory genes is increased in adipose tissue derived from obese mice or obese humans (141, 147). Macrophages infiltrate obese adipose tissue and, in obese mice, can constitute upwards of 50% of the cells isolated from the adipose tissue (141). These cells are the source of the bulk of the TNF-α expression observed in adipose tissue (141). Indeed, with the exception of adiponectin and leptin, nonadipocyte cells are the source of most of the adipokines released from human adipose tissue (31). Other serum factors that are elevated in obesity may derive from effects of these cytokines on the vasculature (122). Importantly, these cytokines and chemokines correlate with the presence of diseases common to obesity, including Type 2 diabetes, hypertension, and atherosclerosis (4, 99, 132, 140), suggesting that the inflammation is functionally important.

There are also obesity-related changes in other adipokines (proteins synthesized and released from adipose tissue), including hormones involved in energy regulation, such as leptin and resistin, as well as other factors, such as angiotensinogen and VEGF (Fig. 4) (7, 23, 37, 109, 120). In contrast, serum levels of the adipokine, adiponectin, an anti-inflammatory hormone, are decreased in obesity (29, 111, 149). Even though the liver is considered the main source of acute phase reactants such as serum amyloid A and C-reactive protein, adipose tissue also produces many of these reactants and appears to be the source of the increases in some of these reactants that are observed in Type 2 diabetes (76). The observation that mice rendered adipocyte deficient have a marked decrease in their ability to increase serum amyloid A and IL-6 following injection of endotoxin also indicates that adipose tissue is an important source of acute-phase proteins (96).

Many of the adipokines listed in Fig. 4 have been associated with asthma, and it is conceivable that increases in their expression in the obese state could exacerbate airway inflammation or airway obstruction in asthma. For example, serum eotaxin is increased in obese humans and in mice with diet-induced obesity (138). Similarly, serum levels of eotaxin are elevated in *ob/ob* and *db/db* mice (unpublished observations). Experiments in eotaxin-deficient mice demonstrate that eotaxin contributes to the eosinophilia observed following allergen sensitization and airway challenge (106). VEGF, a potent angiogenesis factor, is expressed in adipose tissue (31), and VEGF is elevated in the serum of overweight and obese individuals (120). In asthmatic subjects, the number of airway cells expressing VEGF correlates with airway vascularity and correlates inversely with airway caliber (47). Obesity-related increases in serum plasminogen activator inhibitor-1, an important endogenous inhibitor of fibrinolysis and plasmin activation, could predispose toward AHR through effects on extracellular matrix turnover, as previously discussed (114, 115). Visfatin, previously described as pre-B-cell colony-enhancing factor, is an insulin mimetic that predisposes vascular smooth muscle toward a contractile phenotype (136). A similar effect of visfatin on airway smooth muscle, in conjunction with increased serum concentrations of visfatin in obesity (37), could lead to AHR.

TNF-α

As previously described in detail (115), there is reason to believe that obesity-related increases in circulating TNF-α may contribute to the relationship between obesity and asthma. A recent report indicates that peripheral blood monocytes from patients with refractory asthma have increased expression of membrane-bound TNF-α, TNFR1, and TNF-α-converting enzyme (10). Compared with placebo, asthmatic subjects in whom TNF-α was blocked by treatment with etanercept, a soluble TNF-α receptor, also showed reductions in airway responsiveness, increases in forced expiratory volume in 1 s, and improvements in asthma quality of life scores (10). Anti-TNF-α antibody also reduces allergen-induced increases in methacholine responsiveness and pulmonary eosinophilia in a murine model of allergen-induced asthma (63), and the AHR induced by O_3 is also attenuated in TNF-receptor-deficient mice (20, 117). Taken together with the observation that exogenous TNF-α can induce AHR (133), the data suggest that the increase in circulating TNF-α observed in obese mice (49) may contribute to both their innate AHR and their increased responsiveness to O_3. If so, it may be useful to examine the therapeutic potential of etanercept in obese asthmatic subjects.

IL-6

Obesity-related increases in IL-6 may also contribute to the relationship between obesity and asthma (Table 2). The source of much of the IL-6 in the serum of obese subjects appears to be the adipose tissue: measurements of arteriovenous differences across abdominal adipose tissue in obese subjects demonstrate higher levels of sTNFR1 and IL-6 in venous than arterial blood (86), and basal levels of IL-6 are substantially reduced in adipocyte-deficient mice (96). Serum IL-6 correlates with the development of other obesity-related syndromes, including atherosclerosis, hypertension, and diabetes mellitus (17, 100, 102), and polymorphisms of the IL-6 receptor are associated with BMI (30, 145). Treatment of mice with recombinant IL-6 also promotes the development of fatty streaks in the aortic sinus, suggesting that IL-6 is more than just a marker of atherosclerosis, but is required for early lesion development (51). We and others have previously reported that IL-6 is required for some aspects of O_3-induced pulmonary inflamma-

Table 2. *Evidence supporting a role for IL-6 in the relationship between obesity and asthma*

IL-6 and obesity
Increased IL-6 and sIL-6R in obese humans and/or obese mice
Exogenous administration of IL-6 promotes atherosclerosis
Polymorphisms of the IL-6R are associated with obesity
Serum IL-6 correlates with the development of obesity-related syndromes
IL-6 and asthma
Anti-IL-6 antibodies attenuate airway responsiveness in obese mice
Anti-IL-6 antibodies partially ablate obesity-related differences in the response to O_3
IL-6 and sIL-6R are increased in asthma
Blockade of sIL-6R suppresses Th2 cells in a murine model of allergic airways disease

sIL-6R, soluble IL-6 receptor; Th2, T helper 2.

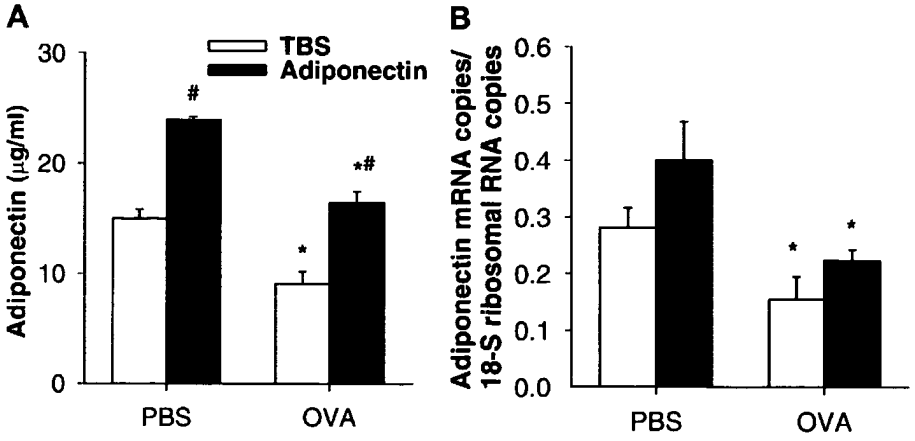

Fig. 5. Effect of treatment with Tris-buffered saline (TBS) buffer or adiponectin on serum adiponectin (*A*) and adipose tissue adioponectin mRNA expression (*B*). Mice were challenged with either phosphate-buffered saline (PBS) or ovalbumin (OVA). Results are means ± SE of data from 4–15 mice in each group. Adiponectin mRNA expression was normalized for expression of 18S rRNA. $*P < 0.05$ vs. PBS group with same treatment; $\#P < 0.05$ vs. TBS-treated mice with same aerosol challenge. [Reprinted from Shore et al. (119) with permission of the American Academy of Allergy, Asthma and Immunology.]

tion (59, 154), suggesting that obesity-related increases in IL-6 may also contribute to the exaggerated responses to O_3 observed in obese mice. Indeed, preliminary data from our laboratory demonstrate that anti-IL-6 antibody attenuates some, but not all, aspects of the increased O_3-induced inflammation observed in *ob/ob* mice (68). Surprisingly, we also observed reductions in innate airway responsiveness in obese, but not lean, mice treated with anti-IL-6 antibodies. Serum concentrations of the soluble IL-6 receptor are elevated, both in obesity (153) and in asthma (28, 105). IL-6 expression is also increased in the airways of asthmatic subjects (84). Increased soluble IL-6 receptor can combine with IL-6 and with membrane-bound gp130 to induce IL-6 responsiveness in cells that do not bear the IL-6 receptor (28, 105), suggesting that IL-6 may have effects in the obese state not normally observed in lean individuals.

Leptin

Leptin has profound effects on satiety and metabolism, as described above. However, leptin is also proinflammatory. Leptin is induced by infectious and inflammatory stimuli (41) and, in turn, stimulates proinflammatory cytokine production from monocytes and macrophages (38, 78, 83). Leptin also causes chemotaxis and release of reactive oxygen species in neutrophils (13). Since serum leptin concentrations are markedly increased in obesity (12, 23, 60, 81, 92), it has been hypothesized that these proinflammatory effects of leptin may be relevant to the increased prevalence and incidence of asthma observed in the obese. Two studies have reported an association between serum leptin and asthma (42, 123). Guler et al. (42) noted that serum leptin was predictive of asthma in boys, even after adjusting for BMI. Sood et al. (123) observed higher leptin levels and higher BMI in asthmatic vs. nonasthmatic women. Interestingly, adjusting for serum leptin levels did not affect the association between BMI and asthma in this population (42), suggesting that the relationship between obesity and asthma is not mediated via leptin. Instead, leptin appears to be a predictor of asthma, independent of obesity. Inflammatory cytokines such as TNF-α and IL-1β have been shown to induce the release of leptin from adipocytes (41), and it is conceivable that systemic manifestations of ongoing airway inflammation in asthmatic subjects lead to increased release of leptin, explaining the association between asthma and leptin. Indeed, we have reported that allergen challenge to the airways of sensitized mice increases serum leptin (118).

To address the potential role of leptin in the relationship between obesity and asthma, we implanted microosmotic pumps subcutaneously in lean OVA-sensitized mice, providing a constant infusion of leptin (118). This treatment resulted in an approximate twofold increase in serum leptin compared with mice with pumps delivering saline. The mice were then challenged with aerosolized OVA for several days. When the mice were treated with saline in the pumps, OVA challenge resulted in AHR, an increase in BAL eosinophils, and increases in BAL and lung Th2 cytokine expression. Leptin treatment augmented OVA-induced AHR, even though it did not affect OVA-induced eosinophil influx or Th2 cytokine expression. Taken together, the results suggest that leptin is capable of augmenting allergen-induced AHR through a mechanism that does not involve changes in Th2 cytokines (118). Other data from the literature support the hypothesis that any role for leptin in the relationship between obesity and asthma is unlikely to be mediated through amplification of typical Th2 mechanisms. Leptin has been shown to increase proliferative responses of $CD4^+$ T cells to mitogens and to alter T-lympho-

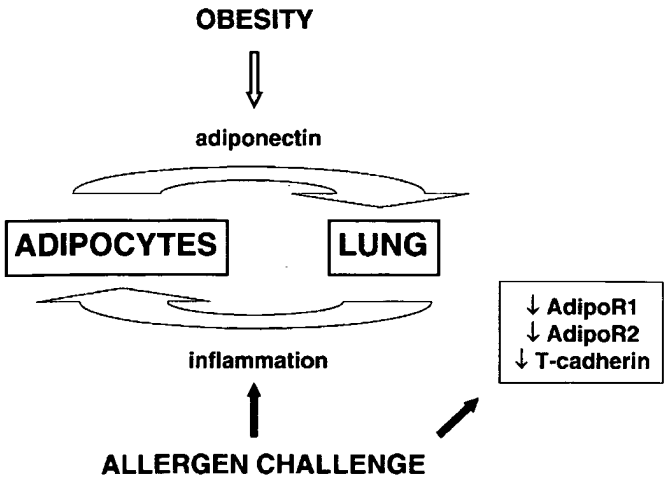

Fig. 6. Schematic representation of interactions between obesity, adiponectin, and allergic airways disease. Open arrows indicate inhibitory effects. See text for details.

cyte cytokine production (80), but the effect is different for T helper type 1 (Th1) and Th2 cells. Leptin increases Th1 cytokine production. Indeed, a recent report indicates a higher percentage of $CD4^+$ T cells secreting IFN-γ in the blood of obese vs. lean children and an association of these cells with serum leptin (95). In contrast, there was no effect of obesity on IL-4 secreting (i.e., Th2) cells. Indeed, leptin has been shown to decrease Th2 cytokine expression (80).

If leptin does play a role in the relationship between obesity and asthma, it is more likely to be mediated through effects on the innate rather than the adaptive immune system. For example, exogenous administration of leptin to lean mice before acute O_3 exposure increases some aspects of their subsequent inflammation response (116). As described above, acute exposure to O_3 primarily induces release of acute-phase cytokines and chemokines. To determine whether even endogenous levels of leptin can impact responses to O_3, we fasted mice overnight before O_3 exposure. Fasting caused a marked reduction in serum leptin, but did not reduce the ensuing inflammatory response. Four hours after acute O_3 exposure (2 ppm for 3 h), BAL protein, IL-6, eotaxin, MIP-2, KC, sTNFR1, sTNFR2, and neutrophils were all increased compared with air-exposed mice, but, with the sole exception of MIP-2, levels of each of these inflammatory markers were the same in fed and fasted mice (61). These results suggest that leptin-related changes in the inflammatory response to O_3 require increases in leptin above those normally observed in lean mice. Such increases are observed in obesity.

Adiponectin

In contrast to other adipokines, plasma adiponectin and adipose tissue adiponectin expression decline in obesity and rise again following weight loss (29, 111, 149). Obesity-related changes in adiponectin are likely to be functionally important, since exogenous administration of adiponectin protects obese mice against obesity-related diseases, including Type 2 diabetes and atherosclerosis (148). Like leptin, adiponectin has profound effects on energy metabolism. Adiponectin acts primarily in the liver and in skeletal muscle to increase glucose uptake, to inhibit gluconeogenesis, and to augment fatty acid oxidation (22, 148, 149). Like leptin, adiponectin also has effects on hematopoietic cells, indicating a role in immunity, but, unlike leptin and other adipokines, adiponectin is anti-inflammatory. For example, adiponectin reduces TNF-α-induced NF-κB activation in endothelial cells (94) and decreases LPS-induced TNF-α production in macrophages (152). Adiponectin has also been shown to increase expression of certain anti-inflammatory moieties, including IL-10 and the endogenous IL-1 receptor antagonist (146).

To test the hypothesis that loss of the anti-inflammatory effects of adiponectin in obesity may play a role in the relationship between obesity and asthma, we implanted mini-Alzet pumps subcutaneously in lean OVA-sensitized mice. The pumps provided a continuous infusion of full-length murine recombinant adiponectin that resulted in an ~50% increase in adiponectin vs. mice implanted with pumps delivering buffer. When the mice were subsequently challenged with aerosolized OVA, there were increases in airway responsiveness, in BAL eosinophils, and in BAL and lung Th2 cytokines in the buffer-treated mice, but these changes were either markedly attenuated or completely absent in mice treated with adiponectin (119). We also observed a decrease in serum adiponectin resulting from reduced adipose tissue adiponectin mRNA expression in mice challenged with OVA (Fig. 5), indicating that allergen challenge in the lung negatively impacts the release of adiponectin from adipocytes. Coupled with declines in mRNA expression of the three currently identified adiponectin binding proteins, adipoR1, adipoR2, and T-cadherin, that were observed in lungs of OVA-challenged mice, the results indicate multiple interactions between the obese state and allergic airways disease involving adiponectin (Fig. 6). Importantly, adiponectin has the potential to negatively regulate allergic inflammation in the lungs. However, allergen challenge also impacts both the levels of circulating adiponectin and the ability of the lung to respond to this adipokine. Coupled with obesity-related declines in serum adiponectin, the obese asthmatic subject is likely to have defects in this important immunomodulatory pathway that augment the effects of allergen challenge.

In conclusion, epidemiological data and studies of the effects of weight gain or weight reduction suggest a causal link between obesity and asthma. These data are supported by reports of innate AHR and increased responses to common asthma triggers in obese mice. There are several biologically plausible mechanisms that could explain a relationship between obesity and asthma. While data from animal models do not allow us to refute the possibility that common etiologies, comorbidities, or mechanical factors play a role in this relationship in humans, they suggest that other factors, possibly adipokines, are also likely to be important. Further understanding of the mechanistic basis for the relationship between obesity and asthma may lead to new therapeutic strategies for treatment in this population.

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Review

High-fat Diets: Modeling the Metabolic Disorders of Human Obesity in Rodents

Roland Buettner, Jürgen Schölmerich, and L. Cornelius Bollheimer

Abstract

BUETTNER, ROLAND, JÜRGEN SCHÖLMERICH, AND L. CORNELIUS BOLLHEIMER. High-fat diets: modeling the metabolic disorders of human obesity in rodents. *Obesity.* 2007;15:798–808.

Research Methods and Procedures: High-fat (HF) diet feeding can induce obesity and metabolic disorders in rodents that resemble the human metabolic syndrome. However, this dietary intervention is not standardized, and the HF-induced phenotype varies distinctly among different studies. The question which HF diet type is best to model the metabolic deterioration seen in human obesity remains unclear. Therefore, in this review, metabolic data obtained with different HF diet approaches are compiled. Both whole-body and organ-specific diet effects are analyzed.

Results: On the basis of these results, we conclude that animal fats and ω-6/ω-9-containing plant oils can be used to generate an obese and insulin-resistant phenotype in rodents, whereas fish oil-fed animals do not develop these disorders.

Discussion: Looking at the present data, it does not seem possible to define an ideal HF diet, and an exact definition of diet composition and a thorough metabolic characterization of the HF diet effects in a researcher's specific laboratory setting remains essential for metabolic studies with this model.

Key words: high-fat diet, metabolic syndrome, animal model

Introduction

Obesity and its associated conditions such as insulin resistance, type 2 diabetes, dyslipidemia, and steatosis hepatis, termed as the metabolic syndrome, represent major challenges for basic science and clinical research. It is obvious that appropriate animals models are crucial for studies on the pathogenesis and therapy of this complex metabolic disorder, but it is less clear how exactly to define the term "appropriate." From a scientific and an ethical point of view, it is reasonable to require that not only the phenotype but also the pathogenesis of the animal's condition resembles the human disease examined. Looking at the polygenic nature of the human metabolic syndrome, it seems that studies examining monogenic [such as the *ob/ob* mouse or the Zucker-(*fa/fa*) fatty rat] or pharmacologically induced (such as the gold-thioglucose mouse model) obesity models must be interpreted with care. The question of whether the results obtained arise from the obese phenotype or the model's genetic/pharmacological background is difficult to answer completely.

For this reason, researchers have been using fat-enriched, so-called high-fat (HF)[1] diets, to generate obese rodent models. The first description of such a nutritional intervention dates back to the 1940s. Subsequent studies have revealed that HF diets promote hyperglycemia and whole-body insulin resistance, and numerous researchers have examined their effects on muscle and liver physiology and on insulin signal transduction. Based on this experience, it is generally accepted that HF diets can be used to generate a valid rodent model for the metabolic syndrome with insulin resistance and compromised β-cell function (1–3). An important downside, however, is the definition of the term "high fat diet" itself. Although more than 650 publications [Medline query using the term "high fat diet AND (rat OR mouse) AND (diabetes OR insulin resistance)", October 2005] have used this approach, neither the exact fat content nor the exact fat composition of the diets employed is standardized. A multitude of different HF diets have been used with relative fat fractions between 20% and 60% energy as fat, and the basic fat component varies between animal-derived fats, e.g., lard or beef tallow, and plant oils,

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[1] Nonstandard abbreviations: HF, high fat; IRS-1, insulin receptor substrate-1; IMCL, intramyocellular lipid; 11-β-HSD-1, 11-β hydroxysteroid dehydrogenase type 1.

e.g., corn or safflower oil. Importantly, many researchers have employed well-defined, semi-purified HF diets, in which the fat component replaces carbohydrate and/or protein, but others have simply added fat to a standard rodent chow. This obviously leads to an unbalanced diet composition with respect to all macro- and micronutrients. Consequently, various diets with very different fatty acid compositions are summarized under the term HF diet in the literature. This has inevitably led to a considerable variability in the results reported.

In this review, we will compile metabolic data obtained with the HF diet intervention. To rule out the effects of possibly unbalanced diets, i.e., chow-based HF preparations, we will concentrate on studies using semi-purified HF diets. We will first look at whole-body diet effects and then examine major metabolic organs, i.e., liver, muscle, adipose tissue, and β-cell. By differentiating fat type effects as far as possible, we will try to define whether there is an ideal HF diet to model obesity in rodents.

General Metabolic Effects of HF Diets

Approximately 60 years ago, Samuels described that rats fed with a diet containing 70% energy as fat developed obesity and elevated basal and postprandial blood sugar values (4,5). Such HF diet effects, noticed when the fat content is well above 30% energy (6,7), have been subsequently specified for different animal strains, fat types, and diet lengths. Table 1 shows the range of metabolic changes described under different HF diets in studies from the last 10 years, in which fat type, diet length, and animal strain were stated. Diets with extreme fat contents (>60% energy) and diets using chow-based HF diets were not included because the physiological significance of these results must be questioned. Due to the scarcity of studies using semi-purified, low-fat control diets, we chose not to use this point as an inclusion criterion for the table.

Obesity

Prolonged feeding with fat-enriched diets induces an increase in body weight in susceptible rats in the range of 10% to 20% over standard chow-fed controls. Obesity induction is most effective when the diet is started at a young age and continues for several weeks (8). Body weight gain during the feeding period is gradual (Figure 1). Although an increase in body weight can be appreciated after as little as 2 weeks, the diet-induced phenotype becomes most apparent after several, i.e., more than 4 weeks of HF feeding. Although no consistent fat type effects can be detected when looking at HF diets based on mammal and plant fats (Table 1), some authors have described obesity resistance and less hypertrophy of visceral fat pads when employing fish oil-based diets (see Table 1). This might be connected with an increased lipid oxidation in these animals due to the fish oil-induced activation of peroxisome proliferator-activated receptor α (9,10).

It is known that a certain fraction of the animals subjected to the HF diet will not become obese. Recent studies have demonstrated that obesity-susceptible animals are hyperphagic, possibly due to a central resistance to the anorexigenic action of insulin (11) and a decreased hypothalamic expression of anorexigenic peptides such as α-melanocyte stimulating hormone and cocaine and amphetamine-regulated transcript (12); rats remaining lean on an HF diet eat the same amount of calories as standard chow-fed controls (13). These diet-induced obesity-resistant animals may turn into important models for the genetic basis of weight gain.

Blood Glucose, Insulin Levels, and Insulin Sensitivity

HF diet effects on blood glucose levels are described discrepantly. Normoglycemia, slight hyperglycemia, and the development of type 2 diabetes have been reported with different diet regimes (Table 1). From the data published so far, one can conclude that prolonged, i.e., several weeks, feeding with both animal and plant fat-enriched diets will eventually lead to moderate hyperglycemia in most rat and mouse strains [most widely used are Wistar/Sprague-Dawley for rats and C57BL/6(j/n) for mice]. With the diet types mentioned above, the elevation of fasting glucose levels is usually accompanied by a moderate to distinct increase in fasting plasma insulin levels (Table 1). As with obesity, fish oil-fed animals generally do not develop such signs of systemic insulin resistance (14). Matching with these biochemical parameters, hyperinsulinemic-euglycemic clamp experiments consistently have demonstrated whole-body insulin resistance in animal and plant fat-fed rodents, whereas fish oil feeding was coupled with sustained insulin sensitivity (15,16). Although it has been stated that saturated fats lead to an enhanced development of insulin resistance in the HF setting (17), it should be remembered that the experimental basis for such an assumption is not very strong in rodent models. In the most cited study (18) directly comparing dietary fat types, the diet labeled saturated contained predominantly unsaturated fatty acids (19% energy saturated, 12% energy monounsaturated, and 28% energy polyunsaturated), and other comparative studies have also failed to confirm a clear association between fat saturation and insulin action (e.g., 19). Also, in our own study, we were not able to detect significant correlations between the saturation level of free fatty acids and insulin action as estimated from whole-body glucose disposal after an insulin challenge (Figure 2).

Insulin resistance was shown after as little as 2 weeks of a lard-based HF diet, indicating the rapid dietary induction of this disorder (20). The development of overt diabetes in these animals is controversial. In our personal (unpublished) experience, some Wistar rats held on a lard-based diet for more than 12 months show fasting glucose levels above 150 mg/dL and postprandial glucose levels of more than

Table 1. Systemic effects of HF diets in rodents

	Citation	Rodent type/strain	Fat type	Energy percentage from fat	Diet length (days)	Effect (%)
Weight	100	Wistar rats	Lard	60	120	+15
	101	Wistar rats	Lard	58	42	+10
	102	Balb/cJ mice	Milk fat	42	136	+11
	103	Long-Evans-rats	Butter	43	75	+10
	81	Lewis rats	Coconut fat	40	70	+16
	104	C57Bl/6 mice	Coconut fat	42	105	+8
	81	Lewis rats	Olive oil	40	70	+8
	104	C57Bl/6 mice	Corn oil	42	105	+11
	81	Lewis rats	Safflower oil	40	70	+19
	1	Wistar rats	Safflower oil	59	21	NC
	23	Wistar rats	Safflower oil	60	300	+28
	81	Lewis rats	Fish oil	40	70	+21
	98	Wistar rats	Fish oil	48*	28	NC
	105	Fischer rats	Fish oil	45	28	−8
Serum glucose	101	Wistar rats	Lard	58	42	+11
	106	C57Bl/6 mice	Milk fat	42	50	+14
	1	Wistar rats	Safflower oil	59	21	+11
	107	Wistar rats	Safflower oil	45	14	+15
	23	Wistar rats	Safflower oil	60	300	NC
	108	Wistar rats	Safflower oil	58	28	+26
	108	Wistar rats	Fish oil	58	28	+24
	98	Wistar rats	Fish oil	48*	28	NC
	105	Fischer rats	Fish oil	45	28	−5
Serum insulin	109	Wistar rats	Lard	25	21	+81
	110	129S1 mice	Lard	45	98	+100
	100	Wistar rats	Lard	60	120	−18
	101	Wistar rats	Lard	58	42	NC
	103	Long-Evans-rats	Butter	43	75	+35
	106	C57Bl/6 mice	Milk fat	42	50	+510
	1	Wistar rats	Safflower oil	59	21	+20
	23	Wistar rats	Safflower oil	60	300	+170
	107	Wistar rats	Safflower oil	45	14	+85
	108	Wistar rats	Safflower oil	58	28	+95
	108	Wistar rats	Fish oil	58	28	+80
	98	Wistar rats	Fish oil	48*	28	−45
	105	Fischer rats	Fish oil	45	28	−50
Serum triglycerides	101	Wistar rats	Lard	58	42	+90
	81	Lewis rats	Coconut fat	40	70	+175
	81	Lewis rats	Olive oil	40	70	+190
	81	Lewis rats	Safflower oil	40	70	NC
	23	Wistar rats	Safflower oil	60	300	−25
	81	Lewis rats	Fish oil	40	70	−35

Table 1. Continued

	Citation	Rodent type/strain	Fat type	Energy percentage from fat	Diet length (days)	Effect (%)
Serum fatty acids	110	129S1 mice	Lard	45	98	NC
	101	Wistar rats	Lard	58	42	+100
	100	Wistar rats	Lard	60	120	−41
	81	Lewis rats	Coconut fat	40	70	+87
	81	Lewis rats	Olive oil	40	70	+112
	81	Lewis rats	Safflower oil	40	70	+19
	23	Wistar rats	Safflower oil	60	300	−30
	81	Lewis rats	Fish oil	40	70	+9
Leptin	103	Long-Evans-rats	Butter	43	75	+66
	106	C57BL/6J mice	Milk fat	42	50	+1560
Adiponectin	111	Wistar rats	NA	58	21	NC
	110	129S1 mice	Lard	45	98	−11
	80	129/Sv-C57BL6 mice	Lard	40	105	−5
Resistin	112	C57BL/6J mice	Milk fat	42	136	+50

NC, no change; HF, high fat. Shown are exemplary studies highlighting the range of changes observed. Inclusion criteria were: the composition of both HF and control diets had to be detailed (including the fat source) in the paper's methods section; the HF diet was semi-purified, not chow-based, as far as could be judged from the description; the HF diet and the control diet had to be balanced with respect to protein content (we accepted a difference in protein content of up to 5% energy); and the same rodent strain had to be used for the dietary and the control intervention. Due to the scarcity of studies using semi-purified control diets, we chose not to use this point as an inclusion criterion for the table. The data are grouped by fat type (lard, butter, milk fat, mainly saturated and monounsaturated fatty acids; coconut fat, saturated fatty acids, mainly C_{12} to C_{14}; olive oil, monounsaturated fatty acids; safflower and corn oil, polyunsaturated ω-6 fatty acids; fish oil, polyunsaturated ω-3 fatty acids).

* Seven percent fish oil.

300 mg/dL; therefore, these animals can be classified as diabetic. We have not found reliable predictors of this development, which makes it difficult to use this approach for diabetes modeling. Other researchers have described similar results, e.g., with high-olive oil diets in C57BL/6J mice (21,22), whereas Chalkley et al. (23) were not able to induce overt diabetes by HF feeding of Wistar rats with a safflower oil-based diet.

Obesity-Prone and -Resistant Rodent Strains

When looking at the literature, it is obvious that the diet effects depend not only on the diet composition but also considerably on the rodent type and rodent strain. Rats have some advantages over mice when performing metabolic studies due to their larger size (e.g., catheter techniques, blood drawing, etc.). Due to the large number of reported studies, it seems justifiable to see Wistar and Sprague-Dawley outbred rats as the standard rodents for this experiment type. These strains are susceptible to diet-induced obesity and insulin resistance with individual variations. Looking at strain differences with respect to HF diet effects, metabolic research has focused on the dietary fat preferring obesity-susceptible Osborne-Mendel rat and the obesity-resistant (i.e., the carbohydrate preferring) S5B/Pl rat (24,25). The phenotypic variations between these two inbred strains might be due to an altered hypothalamic gene expression (26), leptin sensitivity (27), sympathetic stimulation (28), or epigenetic programming (29).

The analysis of strain-dependent diet susceptibility has been performed more extensively in mice. From this, it is known that the inbred mouse strains C57BL/6J, AKR/J, and DBA/2J are more prone to develop obesity and insulin resistance than SWR/J, A/J, and 129S6 (21,30–37). These strain differences have been used to elucidate the interactions among nutritional factors, behavior, and genetic background. For example, obesity-resistant mice accumulate visceral fat only under a pronounced HF diet containing minimal protein contents (35) and prefer, when given the choice, carbohydrates over fat (38,39). In contrast, obesity-prone mice strains become obese under nearly all (ad libi-

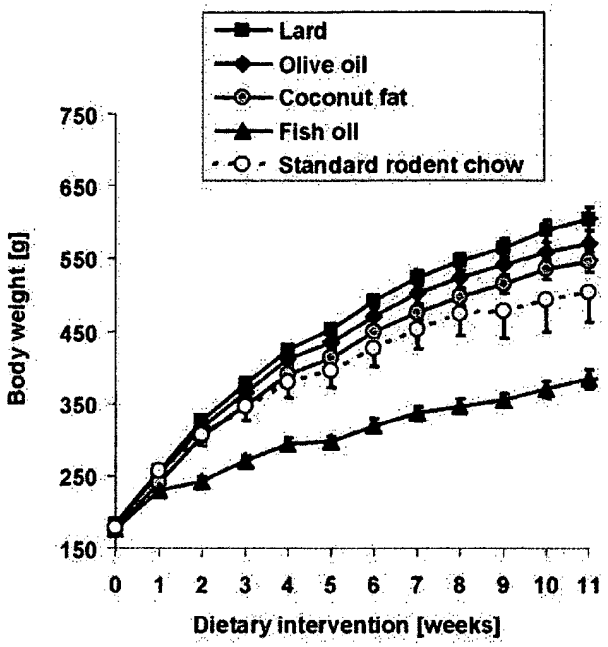

Figure 1: Weight gain and dietary fat type in HF-fed rats. Male Wistar rats were fed with semi-purified HF diets (42% energy from fat, 39% energy from carbohydrate, 19% energy from protein; fat sources: lard, black boxes; olive oil, black rhomboids; coconut fat, gray circles; fish oil, black triangles) or standard rat chow (11% energy from fat, 61% energy from carbohydrate, 28% energy from protein; white circles). The weekly weight gain is shown for the first 11 diet weeks. * $p < 0.05$ when comparing weight gain with standard rat chow ($n = 6$ per diet group).

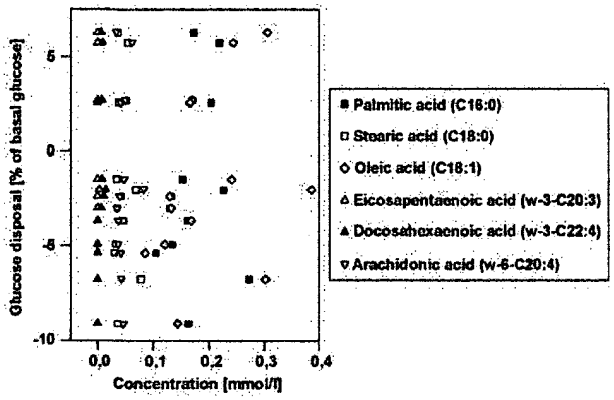

Figure 2: Correlation between plasma free fatty acid subtypes and insulin action. The plasma free fatty acid composition was analyzed in male Wistar rats after a 12-week dietary intervention with different HF diets (42% energy from fat, 39% energy from carbohydrate, 19% energy from protein) ($n = 6$ per diet group). The absolute plasma concentrations of the main saturated fatty acids (palmitic acid, black boxes; stearic acid, white boxes), the main monounsaturated fatty acid (oleic acid, gray rhomboids), the main ω-3 polyunsaturated fatty acids (eicosapentaenoic acid, white triangles; docosahexaenoic acid, black triangles), and the ω-6 polyunsaturated arachidonic acid (gray inverted triangle) in each rat were correlated to the respective animal's whole-body glucose disposal calculated from an insulin tolerance test. No significant associations could be detected.

tum) HF conditions. From these strains, C57BL/6J mice have been employed particularly often as a model for the development of a metabolic syndrome (40) and altered insulin secretion (41). Their proneness toward obesity has been explained by an increased leptin resistance when compared with A/J mice (42,43). A recent genetic study comparing C57BL/6J and 129S6 mice has described a quantitative trait locus on chromosome 14 that contains two genes linked to adipose tissue development and insulin sensitivity, namely Wnt5a and protein kinase C_δ (44).

Lipids

Comparable with the effect on blood glucose levels, most authors report fasting hypertriglyceridemia when using HF diets based on animal fats and plant fats (Table 1), whereas fish oil-based chow tends to lower plasma triglycerides. Data on total cholesterol levels under HF diets are inconsistent, and definite statements about the putative induction of hypercholesterinemia by a pure HF diet, i.e., without addition of cholesterol, do not seem possible from the literature at present. The only exception is fish oil-based diets; there, a hypocholesterolemic effect is clearly established (45–48).

Adipokines

Adipokines such as leptin, adiponectin, or resistin are recognized as systemic factors influencing insulin sensitivity. From the studies published so far (Table 1), these parameters mostly mirror human obesity in HF-fed rats. Leptin and resistin levels tend to be elevated, and adiponectin levels are slightly down-regulated in dietary obese rodents. However, because of the small number of studies giving detailed descriptions of the diet composition, fat type effects cannot be accurately specified.

When summarizing the data presented about general HF diet effects, it seems justifiable to conclude that HF feeding with animal- or plant-derived fats for several weeks leads to obesity, (moderate) hyperglycemia and hyperinsulinemia, hypertriglyceridemia, and changes in the plasma adipokine pattern resembling human obesity and insulin resistance in susceptible animals. Wistar and Sprague-Dawley rats and C57BL/6 mice are the rodent strains used most frequently for this model. Looking at the specific fat type, animal-derived fats such as lard and beef tallow and also plant fats rich in unsaturated ω-9 and ω-6 fatty acids induce the obese phenotype; diets containing a large fraction of marine ω-3 polyunsaturated fatty acids do not. Although these rules apply in general, it is important to remember that diet outcomes vary in different laboratories due to animal strain and diet variations. This implies that a thorough character-

ization of the specific HF model used should be done before examining the effects of any intervention.

HF Diets and Skeletal Muscle

A hallmark of HF diet-induced insulin resistance is an impairment of insulin-stimulated glucose uptake in skeletal muscle in the range of 30% to 60% of standard-fed controls. This effect can be seen already after short periods, i.e., 14 days (20), and with the same fat types that induce whole-body insulin resistance; fish oil consistently does not lead to impaired glucose uptake (15,17,18). The pathophysiology of this effect has been examined closely. Early work demonstrated a reduction of the total amount of insulin receptor without modification of the receptor affinity (49). Subsequent studies have found a decline in insulin receptor autophosphorylation and insulin receptor substrate-1 (IRS-1) phosphorylation (20,50) and a reduced activation of IRS-1-associated phosphatidylinositol-3′-kinase (51). These results suggest that an impairment in the early steps of insulin signaling in HF-fed animals leads to an alteration in the translocation of the glucose transporter-4 (51,52) and, thereby, causes the impairment of insulin-stimulated glucose uptake in muscle. This effect is closely correlated to the accumulation of intramyocellular lipids (IMCLs) induced by HF feeding (18,53), maybe due to a decreased mitochondrial oxidative capacity (54). A reduction of elevated IMCL is associated with an enhanced insulin action in HF rat soleus muscles (55), and interventions directed against the diet-induced increase in IMCL, e.g., overexpression of uncoupling protein 3 in skeletal muscle (56,57) or muscle-specific knockouts of fatty acid transport proteins (58,59), prevent the development of muscle insulin resistance under HF diets. Also, fat-enriched diets have been reported to change the muscle cell membrane phospholipid composition, which might influence insulin binding or glucose transporter-4 function (60,61).

In summary, with the exception of fish oil-based preparations, HF diets increase IMCL and decrease the insulin sensitivity of skeletal muscle by altering early steps of the insulin signal transduction pathway. This closely mirrors the pathogenesis of human obesity-related insulin resistance (62).

HF Diets and Adipose Tissue

It is difficult to define an unanimous phenotype of the HF diet adipocyte, but some changes of adipocyte morphology and metabolism have been found consistently throughout the literature when using animal- and plant-derived HF diets. Adipocyte number and size are increased (63,64), and epinephrine-stimulated lipolysis is reduced (65). An enhanced uncoupling protein expression in brown and white adipose tissue and a decrease in the expression levels of liposynthetic genes (66–68) might provide a partial defense against the HF-induced lipid storage. Interestingly, gene expression profiling has recently revealed an up-regulation of inflammatory genes in adipocytes of diet-induced obese mice (67), which resembles the proinflammatory state described in human obesity.

Animal- and plant-derived HF diets also induce changes in insulin action in adipose tissues. The stimulation of glucose uptake is reduced in brown and white fat (69–71). Decreases in insulin receptor autophosphorylation (72) and an activation of glycogen synthase kinase-3 (73) have been described as molecular basis for these effects, but relatively little is known about the exact changes in adipocyte insulin signaling in this model.

The positive effects of fish oil-based diets on insulin action and obesity in vivo are not completely reflected when looking at isolated adipocytes from these animals. Although experimental data might argue for an attenuation of adipocyte growth and differentiation induced by this diet type, this has not been shown clearly in vivo. At least one study directly examining this question has found fish oil-induced adipocyte hypertrophy and hyperplasia and a reduction of insulin-induced glucose uptake (74), whereas other authors (46,75) have described adipocyte hypoplasia and improved insulin action. Due to these inconsistencies, an unequivocal statement about fish oil effects on adipose tissue function seems premature; more comparative diet studies are needed.

The roles of some adipocyte-typical metabolic pathways in obesity development have been specifically examined with transgenic technologies in HF (mostly lard or safflower oil)-fed animals. Looking at obesity-resistant A/J mice, Morton et al. (76,77) have recently identified the down-regulation of 11-β hydroxysteroid dehydrogenase type 1 (11-β-HSD-1), a major regulator of intracellular glucocorticoid levels, in adipocytes as an obesity-preventing feature in this strain. Consequently, transgenic overexpression of 11-β-HSD-1 in adipocytes leads to a metabolic syndrome in mice, whereas 11-β-HSD-1 knockout animals are resistant to diet-induced obesity. However, this concept of 11-β-HSD-1 as a general factor in obesity development has been questioned recently by findings in obese and insulin-resistant Wistar rats showing no change in adipocyte 11-β-HSD-1 activities after prolonged HF feeding (78).

Animals overexpressing diacylglycerol acyltransferase, the rate-limiting enzyme of triglyceride synthesis, in their adipose tissue become obese under an HF diet but are resistant to diet-induced insulin resistance (79), pointing at the importance of an efficient fat storage in adipose tissue fat depots as a measure against peripheral insulin resistance. Animals lacking hormone-sensitive lipase, the enzyme controlling lipolysis from adipose tissue, remain lean when fed with an HF diet; they also show lower glucose and fasting insulin levels. This was explained by an increase in thermogenesis and energy expenditure and an impaired differentiation of white adipose tissue (80).

Taken together, animal- and plant-derived HF diets lead to adipocyte hypertrophy, hyperplasia, and insulin resistance. The role of adipocytes as important regulators of whole-body energy and glucose metabolism has been confirmed by HF diet interventions in animals with tissue-specific transgenic manipulations. Contrasting its clear beneficial effects in whole-animal lipid metabolism and the skeletal muscle, the impact of fish oil on adipose tissue function remains unclear.

HF Diets and the Liver

It is well established that the obesity-inducing HF diets based on animal and plant fats also lead to hepatic steatosis (81). From clamp studies, it is clear that this condition is associated in vivo with hepatic insulin resistance, i.e., an impairment of insulin's ability to lower hepatic glucose output (for a recent review, see 82), and interventions designed to improve hepatic fat clearance, e.g., the hepatic overexpression of uncoupling protein-1, have demonstrated improvements of whole-body insulin resistance (83). However, insulin signaling studies in HF-fed rats have shown that the classic disorders observed in muscle and fat are not necessarily replicated in the liver. IRS-1 and IRS-2 proteins and their phosphorylation are not altered, and phosphoinositide-3-kinase activity associated with IRS-1 and IRS-2 is increased (81,84). Also, recent studies have demonstrated sustained insulin action in isolated steatotic livers (85) and important regulatory effects of systemic and central nervous factors on hepatic glucose output (86–90), which questions whether insulin resistance is a truly intrinsic trait of diet-induced hepatic steatosis, or may be secondarily enhanced, at least partially, by systemic factors. Very recent work has drawn attention to the activation of nuclear factor κB by HF diets in the liver, which might contribute both to the development of non-alcoholic inflammatory hepatic disease and diet-induced disorders of hepatic glucose metabolism (91). Differing potencies of specific diets to activate hepatic nuclear factor κB, therefore, might explain the somewhat controversial results with respect to hepatic insulin action; to our knowledge, this has not been examined systematically so far.

In conclusion, HF diets can induce hepatic steatosis and signs of hepatic insulin resistance in the whole animal; this closely resembles the human obese state. The possible dietary activation of hepatic inflammatory pathways may be part of the link between diet-induced fat deposition and non-alcoholic steatohepatitis.

HF Diets and the β-Cell

Detrimental effects of free fatty acids on the insulin-producing pancreatic β-cell, the so-called lipotoxicity concept, have been proposed as a major pathomechanism for type 2 diabetes (92–94). However, this effect has mainly been characterized in vitro employing either fatty acid exposed Langerhans islets or immortal β-cell lines, and the pathogenic relevance of lipotoxic effects on the β-cellular insulin metabolism in vivo are less proven. Although HF feeding leads to a β-cellular overflow of free fatty acids (95), fewer than 10 studies have been performed so far regarding β-cellular lipotoxicity in this dietary animal model.

Insulin secretion was assessed by an intravenous glucose tolerance test in C57BL/6J mice after an HF diet containing 58% energy from (not further specified) fat for 1 to 10 months (96). The animals showed a quantitatively unaltered first phase but an increased second phase insulin secretion when compared with controls. Considering the insulin resistance induced in the HF-fed animals, the first phase insulin secretion was, therefore, regarded as inadequately low and interpreted as a sign of an impaired β-cell compensation. The significance of this study in terms of lipotoxicity is limited by the fact that the animals developed a concomitant hyperglycemia due to the dietary intervention (glucolipotoxicity instead of lipotoxicity). Similar methodical limitations also apply to another study in which parameters of insulin metabolism were analyzed ex vivo in isolated islets after a 3-month dietary intervention (97). The isolated islets of the HF animals had stable intracellular insulin stores and expressed more preproinsulin-mRNA in comparison with islets of normally fed controls, but their secretory output under stimulating glucose concentrations (>8.3 mM) was decreased. In a study using Sprague-Dawley rats instead of C57BL/6J mice, a 2-week HF dietary intervention (58% energy from a fat not further specified) led to a blunted insulin secretion under normal (5.6 mM) and stimulating (8.3 mM) glucose concentrations with an altered first phase insulin secretion (2). As a possible sign of lipotoxicity, the preproinsulin-mRNA expression was decreased, which is in clear contrast to the above-mentioned long-term studies in mice (97). The short-term dietary intervention was not accompanied by morphometric differences in terms of diameter and/or size of the collagenase-isolated islets (2). In contrast, a more detailed morphometric analysis of serial sections of pancreata from HF rats revealed a significant increase of both islet and β-cell size when comparing HF animals (containing 42% energy from lard) to animals on a standard chow (3). Such a compensatory increase of islet/β-cell size was interpreted as a response to an increased insulin demand caused by peripheral insulin resistance of HF animals. The data emphasize the difficulties in keeping apart direct lipotoxic effects on the pancreatic β-cell and indirect effects on the pancreatic β-cell provoked by HF diet-induced insulin resistance.

One study investigated different effects of a 4-week intervention with either a conventional fat diet (based on mainly lard and corn oil) or a ω-3 fatty acid-enriched HF diet. Here, the insulin levels in the fish oil-supplemented

animals were inadequately decreased in relation to their concomitant insulin sensitivity, and isolated islets from these animals showed a decreased insulin output. This was interpreted as a possible direct lipotoxic effect that might be caused by the qualitative pattern of serum free fatty acids independent of the degree of insulin resistance and of quantitative fatty acid levels (98).

Finally, a very recent study investigated the impact of an HF diet during pregnancy on the endocrine pancreas of the offspring. The newborn rats showed a decrease of β-cell size and number, whereas α-cells were larger and more numerous than in controls on standard chow (99).

Taken together, HF animals seem to be a useful model to validate the physiological relevance of β-cellular lipotoxicity, but as such, they have been mostly neglected. Until now, there are a few studies with rodents that, indeed, indicate that HF diets might induce a milieu of lipotoxicity and directly (i.e., independent from concomitant insulin resistance) impair the β-cellular insulin metabolism. Lipotoxicity in vivo might, however, not be measured by quantitative means (i.e., hypertriglyceridemia, hyperlipacidemia) but rather by qualitative means (i.e., fatty acid composition), which is dependent on the diet regimen. Here, more comparative studies employing different biochemically defined HF diets are required.

Conclusions

HF diet feeding allows the characterization of obesity development and the evaluation of anti-obesity interventions in an in vivo experimental setting that is pathophysiologically very similar to the human disease. By this, it has contributed immensely to the understanding of diet-induced obesity and insulin resistance and many pathophysiological concepts in the field, e.g., the importance of ectopic fat deposition, the interaction between inflammation and insulin resistance, or the concept of β-cellular lipotoxicity have been further evaluated with this method.

From the literature and our personal experience, it seems appropriate to state that semi-purified diets with a fat content of more than 40% energy based on animal fats and ω-6/ω-9 fatty acid-containing plant oils will lead to obesity and insulin resistance, whereas diets with large amounts of (marine) ω-3-fatty acids will not. However, given the plethora of dietary regimes reported in the literature, it is not possible to define a single unifying HF diet composition. Also, diets theoretically composed of identical fat types might yield different results due to uncontrollable differences between primary fat sources and the diet preparation. Therefore, we recommend that any study using the HF diet concept should employ only nutritionally sound, semi-purified HF diets with a defined macro- and micronutrient composition, clearly detail the diet and the provider in publications, and thoroughly evaluate pure diet effects in the specific laboratory setting. Only by these means can interventional effects be judged correctly, and only then will the HF diet model continue to deepen the understanding of diet-induced metabolic disorders.

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PHYSIOLOGY & BEHAVIOR

Genetic vulnerability to diet-induced obesity in the C57BL/6J mouse: physiological and molecular characteristics

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Abstract

The development of the metabolic syndrome in an increasing percentage of the populations of Western societies, particularly in the United States, requires valid models for establishing basic biochemical changes and performing preclinical studies on potential drug targets. The C57BL/6J mouse has become an important model for understanidng the interplay between genetic background and environmental challenges such as high-fat/high-calorie diets that predispose to the development of the metabolic syndrome. This review highlights metabolic and signal transduction features that are altered during the course of disease progression, many of which mirror the human situation.

Keywords: Diet-induced obesity; Hyperinsulinemia; Hyperlipidemia; Catecholamines

1. Introduction

In 1988, Reaven [1] described the existence of a constellation of metabolic abnormalities that seemed to occur together and be related to the etiology of cardiovascular disease. He termed this clustering of anomalies syndrome X. Syndrome X was defined as the coexistence of hyperinsulinemia, hyperlipidemia, and hypertension in nondiabetic individuals. Together, these features were seen as a risk factor for both cardiovascular disease and Type 2 diabetes. Later, intra-abdominal obesity was added as a risk factor, and Bjorntorp [2] suggested the term metabolic syndrome. More recent research has shown that increased fasting insulin, increased fasting glucose, and elevations in hemoglobin A_{1c} (HbA_{1c}) all predict both the development of Type 2 diabetes and cardiovascular disease. Bjorntorp suggested that the "metabolic syndrome" be called the "civilization syndrome" because the various metabolic components of the syndrome as well as clinical diabetes have been seen to be associated with lifestyle changes associated with Western urbanization. These changes include the increased mechanization of work formerly done by humans as physical activity, increased accessibility and affordability of dense, high-calorie foods, and urban sprawl that increasingly necessitates automobile use instead of walking. In addition to decreased exercise and increased caloric intake, epidemiological observations provide evidence that the development of insulin resistance and Type 2 diabetes is related to fat consumption and negatively related to carbohydrate consumption. To understand the molecular aspects that link these metabolic features, it is important to have appropriate animal models. One of these models that is gaining increasing attention is the C57BL/6J (B6) mouse.

2. Diet-induced obesity in B6 mice

An increase in dietary fat content has been shown to produce diabetes and obesity in various strains of mice [3,4] and in rats [5]. The B6 mouse is a particularly good model of the human metabolic syndrome because it develops a syndrome of obesity, hyperinsulinemia, hyperglycemia, and hypertension, when allowed ad libitum access to a high-fat diet [4], but remains lean and physically normal when restricted to low-fat chow. In marked comparison to B6, other strains, such as the A/J mouse or the C57BL/KsJ (KsJ), are relatively resistant to these effects of a high-fat diet (Fig. 1) [6–8]. The development of insulin resistance, hyperglycemia, and obesity in the B6 mouse closely paral-

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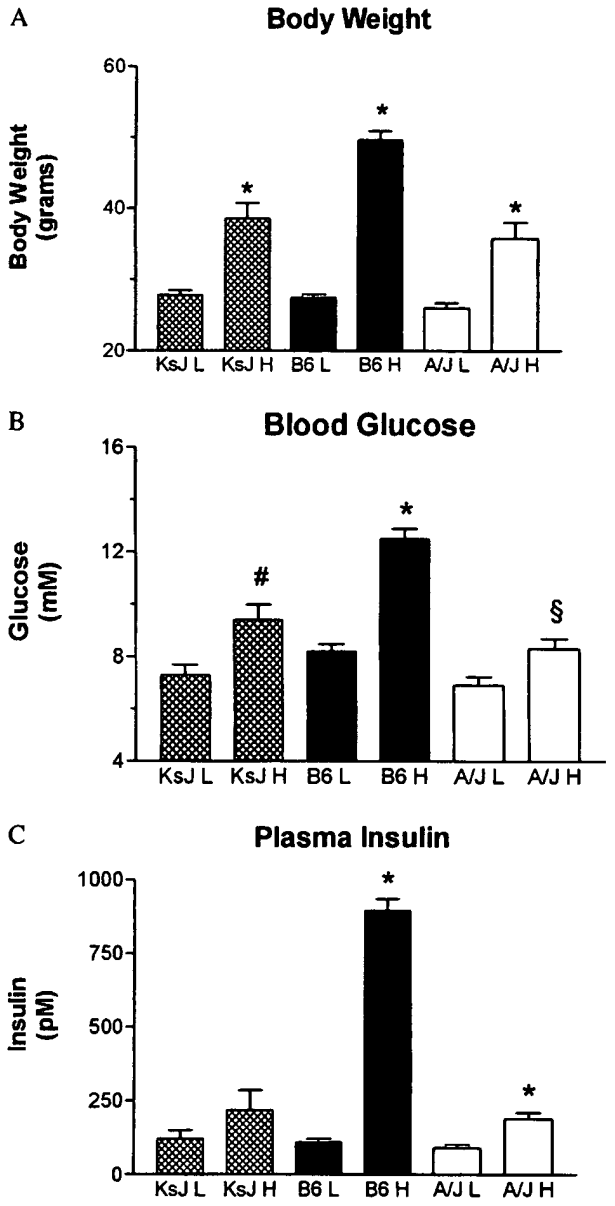

Fig. 1. Effect of a high fat diet on obesity and symptoms of type II diabetes in obesity-resistant and obesity-prone strains of mice. Twenty mice from each of the three strains, A/J, B6, and KsJ, were obtained from The Jackson Laboratory at 3–4 weeks of age and placed on either a low fat diet or a high fat diet as described by Surwit [4]. Body weight (A), plasma levels of glucose (B), and insulin (C) after an 8-h fast were determined after 5 months. One-way ANOVA was performed for each parameter. Body weights, glucose, and insulin values of the low fat-fed animals were not different from each other ($P>0.05$). For all three measures, the values for B6 H mice were greater than those for either the KsJ H or the A/J H mice ($P<0.001$), whereas those for KsJ H and A/J H mice were not different from each other ($P>0.05$). Other statistical comparisons between values for high fat and low fat fed animals are as follows: *, $P<0.001$; #, $P<0.005$; §, $P<0.02$.

lels the progression of common forms of the human disease. For example, the onset of diabetes and obesity in humans occurs gradually and often in the presence of a high-fat diet [9]. In addition, Rebuffe-Scrive et al. [6] and Surwit et al. [7] reported that diet-induced diabetes and obesity in the B6 mouse is characterized by selective deposition of fat in the mesentery, an observation consistent with the finding that abdominal obesity is an independent risk factor for diabetes in humans. Interestingly, others have also noted that high-fat feeding can produce compromised immune function in B6 mice [10], as well as the development of atherosclerosis [11,12].

Obesity in B6 mice fed a high-fat diet is not simply a result of hyperphagia or low levels of physical activity. B6 mice exhibit increased feed efficiency (weight gained/kcal consumed) [7,13,14]. While B6 mice fed a high-fat diet do eat somewhat more than A/J mice [14], this does not account for the additional diet-induced weight gain seen in this strain. In addition, the B6 mouse clearly becomes obese *despite* increased activity levels. Brownlow et al. [13] reported that obese B6 mice are equally as active as their lean counterparts and nearly three times as active as A/J mice. By contrast, Yen and Acton [15] observed that the Lep^{ob}/Lep^{ob} and $LepR^{db}/LepR^{db}$ models are hypoactive. Thus, although the Lep^{ob} mutation is on the same B6 mouse strain, the complete absence of leptin has major effects that are separate from the obesity and diabetes that develops in the nonmutant B6 challenged with high-fat feeding.

The central adiposity that develops in B6 mice is accompanied by metabolic abnormalities. Hyperglycemia develops within 1 month of the introduction of a high-fat diet in the B6 mouse [4,16]. The diabetes/obesity syndrome worsens with time and with increasing obesity. At 16 weeks, B6 mice fed a high-fat diet had developed adipocyte hyperplasia and hypertrophy [17], resulting in animals with a fat mass increased by 93%. As in diabetic, obese humans, the majority of this increased fat mass is found in the mesentery. Although fully manifested after 16 weeks of a high-fat diet, the diabetes/obesity syndrome is completely reversible at this stage in these mice [14]. Like humans with metabolic syndrome, diet-induced obesity and hyperinsulinemia in B6 mice is accompanied by hypertension and increased sympathetic nervous system activity [18]. Finally, similar to euglycemic, young Pima Indians [19], B6 mice also display glycemic responses to stress or to catecholamines, while diet-resistant strains, such as the A/J, do not.

3. Catecholamine signaling and control of adipose tissue metabolism

The sympathetic nervous system plays an integral role in regulating both energy intake and energy expenditure in most mammals. Catecholamines increase lipolysis and decrease triglyceride-rich lipoprotein accumulation in WAT while, at the same time, increasing thermogenesis in BAT, thereby resulting in an overall decrease in total body fat stores [20–22]. The actions of these catecholamines occur via the β-adrenergic receptors (βARs), members of the G-

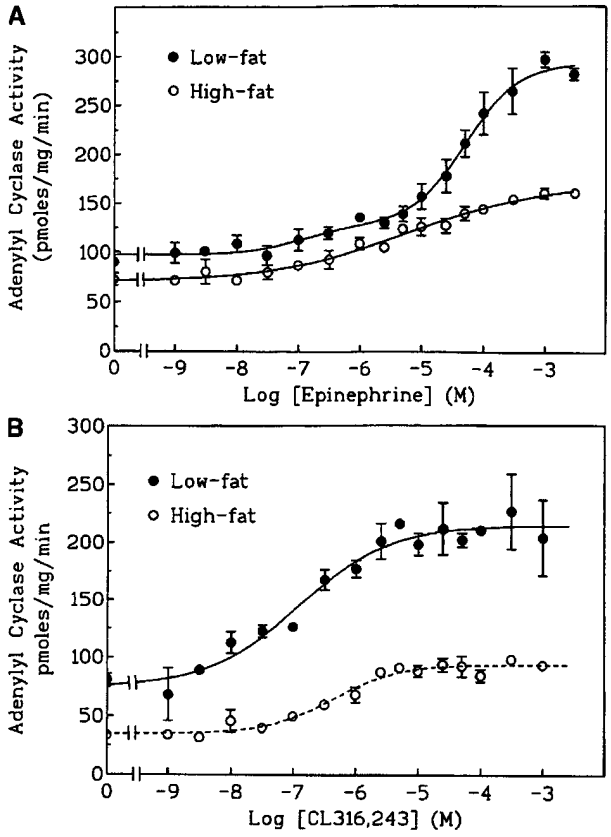

Fig. 2. β-Agonist-stimulated adenylyl cyclase activity in epididymal WAT tissue membranes from B/6J mice. After the 16-week period on one of the three diets, gonadal WAT was removed, and plasma membranes were prepared. β-Agonist-stimulated adenylyl cyclase activity was determined in response to increasing doses of epinephrine (A) or CL316,243 (B). ●, Low fat diet; O, high fat diet.

protein-coupled receptor family. Three βAR subtypes (β_1AR, β_2AR, and β_3AR) have been identified pharmacologically and by molecular cloning of their genes [23–25]. Each of the three βARs is coupled to Gαs and the stimulation of intracellular cAMP levels; however, more complex regulation involving interaction with members of the Gi family has also been reported [26–28]. While all three βARs are expressed in adipocytes, only the expression of the β_3AR is adipocyte specific, and relative levels of expression vary among species [29,30].

In virtually all animal models of obesity, the ability of the βARs to stimulate lipolysis is impaired. Not long after the discovery of the β_3AR, the expression and function of the adipocyte βARs from lean vs. leptin-deficient B6 Lep^{ob}/Lep^{ob} mice were examined. A marked decrease in both β_1AR and β_3AR mRNA levels were observed, whereas the expression of the β_2AR remained unaltered, and these changes in βAR subtype expression were shown to be responsible for the inability to mobilize stored fat in response to β-agonists [31]. Other models of congenital obesity, such as $LepR^{db}$/$LepR^{db}$, tubby, fat, and the Zucker fatty rat, show similar decreases in β_3AR and β_1AR expression [32,33]. The complete absence of βARs in mice created by genetic manipulations [34,35] substantiates these observations and reaffirms the long-standing view that the βARs are the major regulators of catecholamine-stimulated fuel mobilization and thermogenesis. Interestingly, Jimenez et al. [35] observed that the fasting-induced increase in lipolysis was unperturbed in the βAR-less mice. While this raises the interesting possibility of a previously unrecognized signaling system controlling lipolysis under these conditions, compensatory mechanisms may be responsible, for example, the elevated levels of glucagon in these animals. Like these monogenic models of obesity, the B6 mouse raised on a high-fat diet shows similar severe defects in βAR function and expression in adipocytes, while in A/J mice, the decline is less dramatic. This difference between strains is congruent with the mild obesity that develops in A/J [36]. Figs. 2 and 3 illustrate that adenylyl cyclase activity in response to β-adrenergic stimulation is decreased in both white and brown adipose tissue of B6 mice fed a high-fat diet. The expression of the β_1AR and β_3ARs are also severely reduced [36].

Despite these defects in βAR expression in adipose tissue, under certain circumstances selective β_3AR agonists

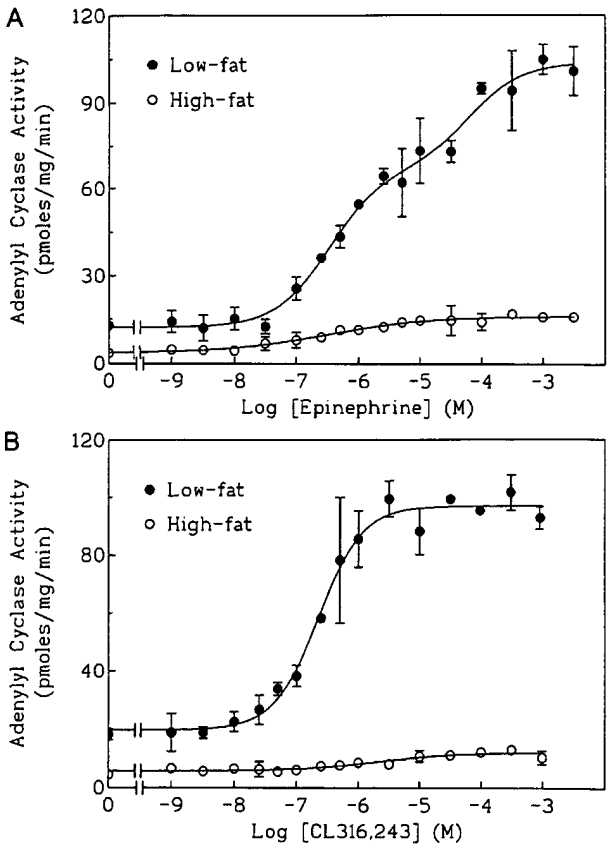

Fig. 3. β-Agonist-stimulated adenylyl cyclase activity in Bat membranes from B/6J mice. After the 16-week period on one of the three diets, IBAT was removed, and plasma membranes were prepared. β-Agonist-stimulated adenylyl cyclase activity was determined in response to increasing doses of epinephrine (A) or CL316,243 (B). ●, Low fat diet; O, high fat diet.

can prevent or reverse obesity and diabetes in a variety of species [36–41]. In addition, the blunted expression of β_3AR appears to be modestly improved, as in the case of the Lep^{ob}/Lep^{ob} mice [42] or nearly normalized in the case of diet-induced obese A/J mice [36]. An important point concerning these studies is that the ability of selective β_3AR agonists to stimulate lipolysis and thermogenesis, and normalize hyperglycemia and hyperinsulinemia, is dependent on genetic background [36,43,44]. As shown in Fig. 4, the effects of β_3AR-agonist treatment can persist over many months. It is also interesting that this ability to prevent weight gain is completely independent of food intake. In fact, as illustrated in the lower panel of Fig. 4, animals consuming the β_3AR-selective agonist in the high-fat diet eat as much, and sometimes more, than animals consuming the high-fat diet alone. In addition, there is no major increase in physical activity that can be observed in these β_3AR-agonist-treated animals (not shown). What is observed however is the dramatic increase in the appearance of brown adipocytes within the retroperitoneal, perirenal, and even epididymal and subcutaneous adipose depots (see Refs. [36,44]).

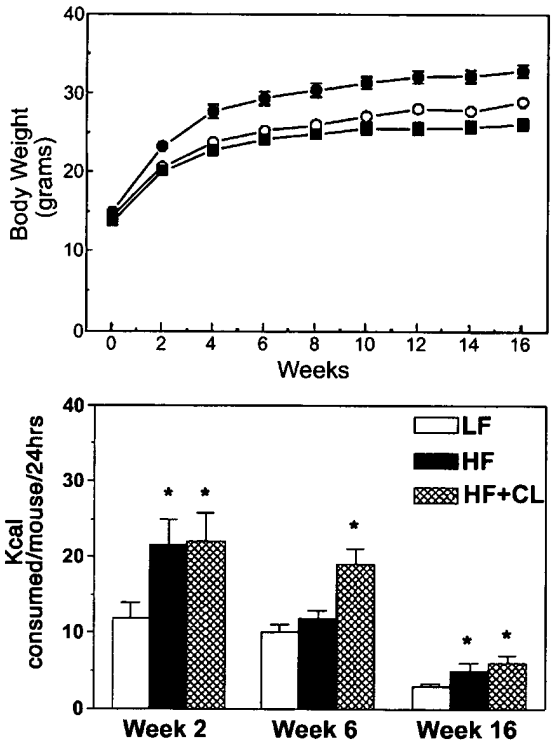

Fig. 4. *Top panel:* Obesity induced by high fat feeding and its prevention by a β_3AR agonist. Thirty A/J male mice were obtained from The Jackson Laboratories at 4 weeks of age. Groups of 10 mice were randomly assigned to a low fat diet (○), high fat diet (●), or high fat diet containing 0.001% CL316,243 (■). Animals were weighed at biweekly intervals through the 16-week period on the diets. *Lower panel:* Food intake measurements over a 24-h period were made for individual animals at three different times during study (2, 6, and 16 weeks on the diets). Each group of 10 animals was housed individually and grams of food consumed over 24 h were measured. Caloric content of each diet was calculated based on 5.55 kcal/g for the high fat diets and 4.07 kcal/g for the low fat diet. #, significantly different from low fat-fed animals at $P<0.05$. *, significantly different from low fat-fed and high fat-fed animals at week 6 and from low fat-fed animals at week 16, $P<0.005$.

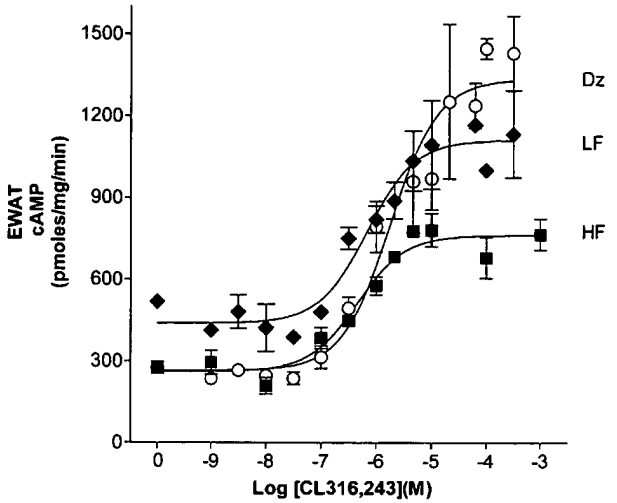

Fig. 5. The stimulation of adenylyl cyclase activity by the β_3AR-selective agonist CL in membranes from animals fed the LF (◆), HF (■), or Dz (○) diets. The assays were incubated for 10 min. The cAMP produced was measured by RIA as described in *Materials and methods.* The data are expressed as picomoles of cAMP produced per mg membrane protein/min incubation. *Curves* represent the mean of three experiments for each condition. Nonliner regression analysis revealed that all three curves were significantly different from each other ($P<0.0003$).

Although genetic and dietary models of obesity display various endocrine abnormalities, the presence of hyperinsulinemia and insulin resistance is the most common variable (discussed in Ref. [45]). In addition to being causally related to obesity, the condition of hyperinsulinemia and insulin resistance may itself lead to obesity. For these reasons, our current hypothesis is that hyperinsulinemia contributes to the inhibition in βAR expression and function in adipocytes. In support of this idea, treatment of 3T3-F442A adipocytes with insulin resulted in a rapid decrease in β_3AR expression [46]. In addition, a role for insulin in affecting βAR function in adipocytes is suggested by two sets of studies. The first showed that suppressing hyperinsulinemia with the K-ATP channel agonist, diazoxide (Dz), results in an improved ability to stimulate lipolysis and a significant loss of adipose tissue mass [47,48]. More recently, we again directly examined the situation in the high-fat fed B6 mouse and showed that treatment with Dz resulted in an increase in the expression and function of the β_3AR, as evidenced by increased cAMP production in response to selective β_3AR agonist stimulation (Fig. 5) [49]. In addition, this increase in β_3AR was accompanied by a significant loss of WAT mass and percent body fat [49]. Importantly, glucose transport into WAT, but not muscle, was increased in response to CL + Dz, suggesting that changes in insulin sensitivity in fat rather than muscle are critical to the development of diabetes, at least in the B6 mouse. It will now be important to extend

these findings to use more selective sulfonyl urea receptor-1 agonists, as well as to investigate at the cellular level how alterations in insulin action at the adipocyte may compromise βAR expression and function.

In summary, the B6 mouse models many of the features of human obesity and metabolic syndrome. When raised on a low-fat diet, the B6 mouse is lean and euglycemic with normal insulin levels and blood pressure. However, when raised on a high-fat diet, animals develop central adiposity, hyperinsulinemia, hyperglycemia, and hypertension. This syndrome appears to be related to abnormalities in adrenergic control of adipocyte function, which, in turn, appear to be related to hyperinsulinemia. Future studies aimed at studying the multifactorial connections between obesity, hyperinsulinemia, and sympathetic nervous system abnormalities may shed light on the insulin resistance syndrome.

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UNITED STATES PATENT AND TRADEMARK OFFICE

**BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES**

Ex parte PAUL P. LATTA

Appeal 2007-1152
Application 10/660,924
Technology Center 1600

Decided: October 10, 2007

Before ERIC GRIMES, LORA M. GREEN, and RICHARD M. LEBOVITZ, *Administrative Patent Judges*.

LEBOVITZ, *Administrative Patent Judge*.

DECISION ON APPEAL

This is a decision on appeal from the Examiner's final rejection of claims 2-9. We have jurisdiction under 35 U.S.C. § 6(b). We reverse.

STATEMENT OF THE CASE

The Specification describes methods of creating immunological tolerance to cells and tissues comprising implanting in the mammal a tolerizing dose of cells or tissue encapsulated in a biologically compatible permselective membrane (Spec. 3). The Specification states that this

process can be used to prevent certain diseases, such as Type I diabetes (Spec. 10: 12-17).

Claims 2-9 are pending and appealed (Supp. Appeal Br.[1] 2). Claims 2-9 stand rejected under 35 U.S.C. § 112, first paragraph, as lacking enablement (Answer 4) and also for lacking written description (Answer 8).

We select claim 2, the only independent claim on appeal, as representative of the claims. It reads as follows:

> 2. A method of preventing onset of Type I diabetes in a mammal predisposed to Type I diabetes, comprising implanting a dose of insulin-producing cells encapsulated in a biologically-compatible membrane into an implantation site in said mammal prior to onset of Type I diabetes, wherein said dose is at least one order of magnitude less than that necessary to achieve normoglycemia in a mammal of the same species.

DISCUSSION

Enablement rejection

"To be enabling, the specification of a patent must teach those skilled in the art how to make and use the full scope of the claimed invention without 'undue experimentation.'" *Genentech, Inc. v. Novo Nordisk, A/S,* 108 F.3d 1361, 1365, 42 USPQ2d 1001, 1004 (Fed. Cir. 1997) (quoting *In re Wright*, 999 F.2d 1557, 1561, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993)). "When rejecting a claim under the enablement requirement of section 112, the PTO bears an initial burden of setting forth a reasonable explanation as to why it believes that the scope of protection provided by that claim is not adequately enabled by the description of the invention provided in the

[1] "Supp. Appeal Br." is a reference to the Supplemental Appeal Brief which is date stamped Jul. 19, 2006.

specification of the application; this includes, of course, providing sufficient reasons for doubting any assertions in the specification as to the scope of enablement. . . . *Marzocchi*, 439 F.2d at 223-24, 169 USPQ at 369-70." *In re Wright*, 999 F.2d at 1561-62, 27 USPQ2d at 1513 (Fed. Cir. 1993).

Enablement is determined as of the application filing date. *See In re Brana*, 51 F.3d 1560, 1567 n.19, 34 USPQ2d 1436, 1441 n.19 (Fed. Cir. 1995). Thus, the issue in this rejection is whether the Examiner has set forth a reasonable explanation as to why the scope of protection provided by the claims is not adequately enabled by the Specification as of the application filing date.

The Examiner contends that the Specification does not adequately teach how to effectively prevent the onset of Type I diabetes in any mammal predisposed to it (Answer 4). The Examiner asserts that the Specification "only discloses the effects of the implanting of insulin-producing cells on the level of blood glucose using streptozotocin-induced [diabetes] in murine experimental model, using NOD mouse. (See Examples 1-2 in particular)" (Answer 4). However, the Examiner contends that the examples are insufficient to enable the scope of the claims because "the state of the art is that it is unpredictable [from] the in vivo murine data using NOD model disclosed in the specification as [to] whether the instant invention can be used for the in vivo preventing onset of type I diabetes in mammals including human" (Answer 7). To support the position that the murine model is not adequate to predict the efficacy of the method as it is broadly claimed, the Examiner cites six literature references: Atkinson (*Nature*, 1999, vol. 5, pages 601-604), Knip (*Acta Paediatr. Suppl.*, 1998, vol. 452, pages 54-62), Metas (*J. of Immunology*, 2004, vol. 172, pages 2731-2738),

Tufveson (*Immun. Reviews*, 1993, No. 136, pages 101-107), Feldman (*Transplant. Proc.*, 1998, vol. 30, pages 4126-4127), and Cochlovius (*Modern Drug Discovery*, October 2003, pages 33-38), and discusses their relevance to the enablement issue (Answer 4-7).

We have considered the Examiner's arguments and the supporting documents, but do not find that the evidence is sufficient to sustain the rejection.

In beginning our analysis, we note that the Examiner did not correctly characterize the disclosure in the Specification. Example 1 describes the treatment of streptozotocin-induced diabetes in C57B6 mice (Spec. 19: 30 to 20: 3), not NOD mice as stated on page 4 of the Answer. The Specification provides experimental evidence in this example that a tolerizing first dose of encapsulated insulin-producing cells induced immunological tolerance to a subsequent curative dose of cells.

Turning to the references cited by the Examiner in support of his position, we first address Mestas (*supra.*). The Examiner states that Mestas provides evidence of the inadequacy of mouse models in predicting the efficacy of therapies for human disease (Answer 5). Mestas – as noted by the Examiner – describes the differences between mouse and human immunology, concluding that "[s]uch differences should be taken into account when using mice as preclinical models of human disease" (Mestas, Abstract). Mestas describes its purpose as "to understand the potential limitations of extrapolating data from mice to humans" (Mestas, at 2731, col. 2). After extensively characterizing the immunological differences, Mestas concludes:

> While caution in interpreting preclinical data obtained in mice is clearly warranted, we believe that with these caveats in mind, mice will continue to be the premiere in vivo model for human immunology and will be absolutely essential for continued progress in our understanding of immune function in health and disease.

(Mestas, at 2736, col. 2.) Thus, far from abandoning the mouse as a model of human disease, Mestas characterizes it as "absolutely essential for continued progress" in understanding immunological diseases.

The Examiner contends that Tufveson's statement that "today's small animal models seem to be insufficient to produce data for clinical decision-making" (Tufveson, at 101) raises reasonable doubt about the predictability of mouse models (Answer 6). However, Tufveson concluded that after "this airing of problems in the clinical field it is clear that small animal models are sought" for human disease (Tufveson, at 101). Tufveson reviewed its own efforts at developing such models and concludes that "it would seem to be reasonable to test new immunosuppressive drugs . . . in allograft models" (Tufveson, at 107). Thus, Tufveson does not disavow the use of animal models nor does it provide any evidence that the particular mouse model described in the Specification is deficient.

With respect to the NOD mouse model of Type I diabetes, Atkinson acknowledges that "specific differences . . . restrict their interpretation" (Atkinson, at 601, col. 2), but also states that "investigations of NOD mice have enhanced our appreciation of the etiologic complexity of type I diabetes in humans and provided an example of how promising results obtained in an animal model can be translated into human clinical trials" (Atkinson, at 604, col. 1). Thus, contrary to the Examiner's position,

Atkinson recognizes that NOD mice – despite species differences – have value in predicting outcome in human disease.

Appellant provided two declarations by Dr. David Scharp ("Declaration of David Scharp, M.D.", dated Nov. 25, 2003, and "Second Declaration of David Scharp, M.D. under 37 C.F.R. § 1.132", dated Apr. 28, 2005) showing that NOD mice who received the treatment described in the Specification were prevented from becoming diabetic (Supp. Appeal Br. 5-6; Declaration of David Scharp, M.D., ¶ 5-8). For example, Scharp showed that 3 out 10 mice who received the tolerizing dose of encapsulated islet cells, prior to the onset of diabetes, remained diabetes-free after eight weeks (Declaration of David Scharp, M.D., ¶ 10). Atkinson states that NOD mouse are the favored model for Type I diabetes (Atkinson, at 601, col. 1), and as discussed above, considered them an important tool for understanding and developing treatments for diabetes (Atkinson, at 604, col. 1).[2] Thus, we find the post-filing evidence in the Scharp Declarations sufficient "to prove that the disclosure was in fact enabling when filed." *In re Brana*, 51 F.3d 1560, 1576, fn.19, 34 USPQ2d 1436, fn.19 (Fed. Cir. 1995).

The Examiner's position appears to be that because mouse models don't always work,[3] they cannot be relied upon to enable a specification –

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[2] Dr. Scharp makes similar comments about the value of NOD mice, including their proven value in developing markers for predicting human patients who will develop clinical Type I diabetes (Second Declaration of David Scharp, M.D. under 37 C.F.R. § 1.132, ¶ 2).

[3] "Mestas . . . teach that there exist significant differences between mice and humans in immune system development . . . [quoting Mestas:] [']As therapies for human diseases become ever more sophisticated and specifically targeted[,] it becomes increasing important to understand the *potential limitations of extrapolating data* from mice to humans. *The*

there being always a degree of uncertainty about whether the treatment will prove to be effective in humans. In our opinion, this is not a reasonable standard by which to measure enablement.

The publications cited by the Examiner acknowledge that there are weaknesses in available mouse models as surrogates for human disease, but these same publications continue to use the animal models to study human disease because the animal models are apparently "'as good as it gets', short of a study in humans" (Atkinson, at 601).

In cases where the utility of claimed compounds were questioned, the Federal Circuit has repeatedly held that in vitro and animal tests, when reasonably correlated with the in vivo activity asserted as the utility, were sufficient to satisfy the utility requirement of 35 U.S.C. § 101. *Cross v. Iizuka*, 753 F.2d 1040, 1051, 224 USPQ 739, 747 (Fed. Cir. 1985). In *In re Brana*, 51 F.3d 1560, 34 USPQ2d 1436 (Fed. Cir. 1995), the PTO had argued that mouse data was insufficient to establish utility for a cancer treatment drug. In rejecting this position, the Federal Circuit wrote:

> The Commissioner counters that such in vivo tests in animals are only preclinical tests to determine whether a compound is suitable for processing in the second stage of testing, by which he apparently means in vivo testing in humans, and therefore are not reasonably predictive of the success of the claimed compounds for treating cancer in humans. The Commissioner, as did the Board, confuses the requirements under the law for obtaining a patent with the requirements for obtaining government approval to market a particular drug for human consumption. *See Scott v. Finney*, 34 F.3d 1058, 1063, 32

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literature is littered with the examples of therapies that work well in mice but fail to provide similar efficacy in humans['] [Mestas, at 271] (emphasis added)" (Answer 5).

> USPQ2d 1115, 1120 (Fed. Cir. 1994) ("Testing for the full safety and effectiveness of a prosthetic device is more properly left to the Food and Drug Administration (FDA). Title 35 does not demand that such human testing occur within the confines of Patent and Trademark Office (PTO) proceedings.").

(*Brana*, at 1567, 34 USPQ2d at1442.) We see no reason why enablement of a method claim whose scope includes humans should likewise require human testing. Thus, in view of *Brana*, we conclude that animal models can be used to establish enablement under § 112, first paragraph of a method claim.

The relevant standard is rather whether the scope of the claims bears "a reasonable correlation to the scope of enablement provided by the specification to persons of ordinary skill in the art." *In re Fisher*, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970). *See also Invitrogen Corp. v. Clontech Laboratories Inc.*, 429 F.3d 1052, 1071, 77 USPQ2d 1161, 1173-1174 (Fed. Cir. 2005). In this case where an animal model serves as the enablement for the claimed method, the proper question is whether it reasonably correlates with the method for which patent protection is sought. On this point, the Examiner has provided no evidence that results obtained with streptozotocin-induced diabetes in C57B6 mice, as described in the Specification, do not reasonably correlate with the scope of claim 2. In contrast, Appellant has provided post-filing evidence, that together with Atkinson's teaching about the acceptability of NOD mouse as a model for diabetes, establishes by the preponderance of the evidence that the Specification as filed is, in fact, enabling. *See In re Oetiker*, 977 F.2d 1443, 1445, 24 USPQ2d 1443, 1444 (Fed. Cir. 1992) ("[P]atentability is determined on the totality of the record, by a preponderance of the evidence.").

The Examiner has provided no other explanation as to why the claim is not adequately enabled for the claimed method of preventing onset of Type I diabetes. Thus, on the record before us, we conclude that the Examiner has not sustained the burden of establishing a reasonable basis to question the scope of enablement of the claimed invention. We reverse the rejection of claims 2-9 for lack of enablement

Written description rejection

Under 35 U.S.C. § 112, first paragraph, the specification must contain a written description of the invention. Thus, when claims are amended during patent prosecution, the claimed invention, in its amended form, must be described in the specification. "An applicant complies with the written description requirement 'by describing the invention, with all its claimed limitations.' *Lockwood v. American Airlines, Inc.*, 107 F.3d 1565, 1572, 41 USPQ2d 1961, 1966 (1997)." *Gentry Gallery v. The Berkline Corp.*, 134 F.3d 1473, 1479, 45 USPQ2d 1498, 1502-1503 (Fed. Cir. 1998).

The Examiner contends that claims 2-9, which were added by an amendment dated April 1, 2004, are not described in the Specification as it was originally filed (Answer 8). The Examiner argues that the Specification "as originally filed only disclosed [a] two-step process" involving tolerizing and curative doses (Answer 12), not a one-step method of "preventing type I diabetes comprising ... implanting a tolerizing dose of insulin-secreting cells" in which the dose is at least one order of magnitude less than a curative dose (Answer 12). We do not agree.

The Specification in its original disclosure describes a one-step process of administering a tolerizing dose of insulin-secreting cells. In the "Summary of the Invention," it is stated:

> One embodiment of the invention is a method of creating immunological tolerance to foreign cells, tissues or organs in a mammal, comprising the step of implanting in the mammal a tolerizing dose of foreign cells or tissue encapsulated in a biologically compatible permselective membrane. The method may additionally comprise the step of administering to the mammal a curative dose.

(Spec. 3-4.)

An original claim of the Specification also describes a single step method:

> 1. A method of creating immunological tolerance to foreign cells, tissues or organs in a mammal, comprising the step of implanting in said mammal a tolerizing dose of corresponding foreign cells or tissue which shed antigens contained in or on said foreign cells [,] tissues or organs, said corresponding foreign cells or tissue being encapsulated in a biologically-compatible permselective membrane.

(Spec. 27.)

The tolerizing dose is characterized in the original Specification as being "one to two orders of magnitude less than the curative dose" (Spec. 4: 26-27) and "one or two orders of magnitude less than a full dose implant" (Spec. 12: 26-27). It is stated in the Specification that the "amount of cells . . . necessary for the initial tolerizing implant will vary" (Spec. 12: 24-25) and "the size of these doses . . . can be optimized" (Spec 12: 30-31). Thus, the Specification describes a dose of one order of magnitude less than a curative dose (i.e., the dose recited in clam 2 which is "necessary to achieve normoglycemia in a mammal of the same species") and that this amount

could be routinely varied. Thus, we conclude that the limitation "wherein said dose is at least one order of magnitude less than that necessary to achieve normoglycemia in a mammal of the same species" is supported by the originally filed Specification.

The Specification also explicitly describes preventing diabetes using a single dose of cells – the method of instant claim 2:

> The process of the invention can also be used to prevent certain diseases, particularly autoimmune disorders. In these cases the process is as follows. First, patients at high risk for the disease or already in the very early phase of the disease are identified. At the critical time of the onset, the process is intervened with the small encapsulated tissue. For example, islets are used for Type I diabetes and collagen is used for arthritis. This implant of foreign tissue immediately diverts the attention of the immune system to the new foreign invader and it begins the process to destroy this new threat. Because of this diversion, the process of self-destruction of desirable tissue that was just beginning is suppressed, then abandoned, then forgotten. It is, in essence, "switched off" and the damage is prevented.

(Spec. 10: 12-21.) It is also stated that implants for prevention of disease can be in tolerizing amounts (Spec 19: 1-15).

In sum, we find that all the limitations of claim 2 are described in the originally filed Specification. Accordingly, the rejection of claims 2-9 as lacking written description is reversed.

CONCLUSION

The rejection of claims 2-9 under 35 U.S.C. § 112, first paragraph, as lacking enablement and written description are

REVERSED.

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